The Journal of Laboratory and Clinical Medicine

VOL. 29 NOVEMBER, 1944 NO. 11

CLINICAL AND EXPERIMENTAL

CHOLINE DEFICIENCY STUDIES IN DOGS

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THE importance of choline in the nutrition of the dog was suggested by the discovery of its lipotropic action on the liver of the depancreatized dog by Best, Ferguson, and Hershey in 1933.[1] Study of its deficiency in normal dogs has awaited the use of purified rations. Thus, Schaefer, McKibbin, and Elvehjem[2] demonstrated a lag in the early growth curve of young puppies fed a purified ration without choline. The addition of choline to this ration resulted in resumption of growth. Fouts[3] fed young puppies and adult dogs on a diet high in fat and low in protein without added choline. With such a diet, he reported a fatty degeneration and cirrhosis of the liver. Hough, Monahan, Li, and Freeman[4] observed an impaired hepatic dye clearance and high serum phosphatase values in dogs fed a diet low in protein and a diet low in methionine. This paper presents in detail some observations in the study of choline deficiency in young puppies.

EXPERIMENTAL

Litters of weanling mongrel puppies were used throughout the experiments. Upon receipt of the puppies at the laboratory, they were dewormed with Buchlorin and magnesium sulfate and placed on a diet of milk and Purina Dog Chow for a week or ten days' observation before the experiments were started. All dogs received subcutaneously 1.5 c.c. per pound of Pitman-Moore antidistemper serum each week.

Preliminary studies have shown inconsistencies in the growth of young puppies placed on the purified ration devised by Schaefer and co-workers.[2] One litter of animals grew well except for a slight lag in the early part of the

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Received for publication, Sept. 8, 1944.

TABLE I

RATIONS USED FOR THE PRODUCTION OF CHOLINE DEFICIENCY

RATION NUMBER	1	2	3	4	5
Sucrose	49.5	47.5	46.5	47.5	59.5
Purified casein (SMA)	6.0	7.0	7.0	7.0	10.0
Extracted peanut meal	30.0	30.0	30.0	30.0	
Extracted peanut flour					15.0
Cottonseed oil	7.0	7.0	7.0	7.0	7.0
Cod-liver oil	3.0	2.0	2.0	2.0	2.0
Salts (Phillips and Hart)	4.0	4.0	4.0	4.0	4.0
K_2HPO_4	0.5	0.5	0.5	0.5	0.5
Abbott's Liver Fraction "X"		2.0			
Wilson's Liver Fraction "L"			3.0	2.0	2.0
	MG. PER 100 GM. RATION	μG. PER KILO OF BODY WEIGHT PER DAY GIVEN ORALLY			
Thiamine chloride	0.2	50	100	100	100
Pyridoxine hydrochloride	0.2	50	100	100	100
Calcium pantothenate	1.5	150	300	300	300
Riboflavin	0.4	75	150	150	150
Nicotinic acid	2.5	350	700	700	700

growth curve. This suggested that the methionine of this ration might be making important contributions to the "methyl pool" of these animals and that it might be desirable to find a source of protein other than casein, one lower in methionine but yet adequate in amino acids. The composition of the basic experimental ration used by us is given in column 1 of Table I. It is a slight modification of the ration used by Engel and Salmon[5] in their studies

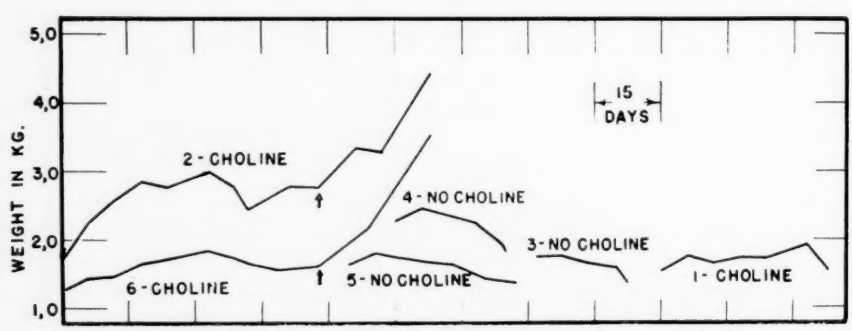

Fig. 1.—Growth curves of puppies of Experiment 1, Ration 1. Arrows indicate inception of supplement of 4 per cent Abbott's Liver Fraction "X" to the control ration.

on choline deficiency in rats and contains about two-thirds of the total protein as peanut protein, which is low in methionine. We had used this basic experimental ration with good results in studies on choline deficiency in rats,[6] and it seemed desirable to use such a ration in studies on the dog. The peanut meal or peanut flour used in these rations was extracted with alcohol and ether to remove choline and choline containing phosphatides. The procedure consisted of one extraction with cold ether followed by six four-hour reflux extractions with hot alcohol.

For the first experiment a litter of six puppies was given Ration 1 (Table I). The growth of these puppies is shown in Fig. 1. Two of the six puppies (Dogs 1 and 2) served as controls and received in addition 200 mg. per cent of choline chloride in the ration. Dog 6 received choline after the ninth day

and also served as a control. It can be seen that this ration did not support growth in either the deficient or the control animals, although control Dog 2 made good progress for the first three weeks. At the end of the first week, oral doses of casein were given to Dogs 3 and 5 and dl-methionine to Dog 1 in an attempt to induce improvement in growth. However, there seemed to be no benefits from these additions. Evidently factors other than protein in nature were still not adequately met by this ration. In further attempts to improve it the following supplements were given to the choline-fed control dogs for varying periods: additional choline chloride; a mixture of the vitamins of the B-complex containing thiamine, pyridoxine, riboflavin, calcium pantothenate, and nicotinic acid; brewers' yeast; and a liver extract, Abbott's Liver Fraction "X." This liver extract fraction was the only supplement that produced any significant improvement. Response to this supplement was rapid and sustained for the duration of the experiment, as is shown in the growth curves of Dogs 2 and 6 in Fig. 1.

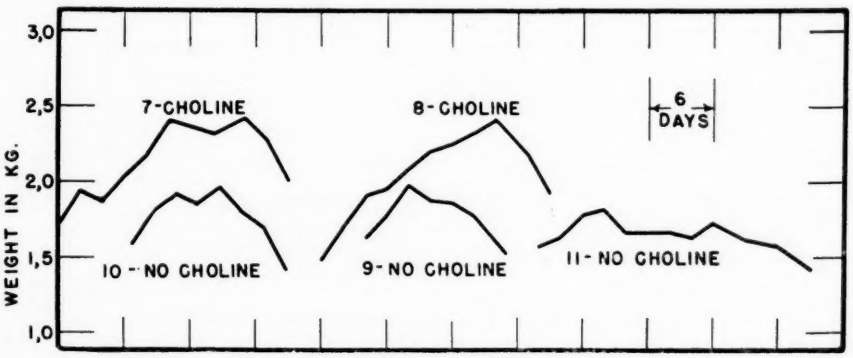

Fig. 2.—Growth curves of puppies of Experiment 2, Ration 2.

With this experience another litter of five puppies (Experiment 2) was placed on Ration 2 (Table I). The growth curves of these puppies are shown in Fig. 2. It is apparent from the growth curves of Dogs 7 and 8, the control dogs receiving choline, that this ration would still not permit survival. The terminal findings for the animals in Experiments 1 and 2 will not be listed in detail since the deficiencies were complicated and have limited meaning in relation to deficiency of choline. It is of interest, however, that all the choline-deficient animals had extremely fatty liver, whereas the liver of the control dogs fed choline appeared normal. The average chloroform-extractable material found in the liver of Dogs 3, 4, 5, 9, 10, and 11 (receiving no choline) was 41.2 per cent. In the liver of Dogs 7 and 8, which had received choline, there was an average of 17.6 per cent chloroform-extractable material.

Experiment 2 indicated that at a concentration of 2 per cent the liver fraction used did not furnish enough of the missing factor or factors to render the control diet adequate. It was decided to try another liver extract low in choline and to use it in slightly higher concentration. The Wilson's Liver Fraction "L" is low in choline and is fairly potent in certain nutritional factors essential for the chick. This liver fraction therefore replaced the original liver fraction used in the ration and was included at a 3 per cent level (Ration

3, Table I). Three litters, totaling twelve puppies, were placed on this ration. The control puppies received in addition 150 mg. per cent of choline chloride. The growth performance of the first two litters (Experiment 3), totaling five puppies, is shown in Fig. 3. It can be seen from the curves that Dog 16 grew well throughout the experimental period of fifty-seven days. The littermate, Dog 15, receiving no choline did not grow at all for the first twelve days.

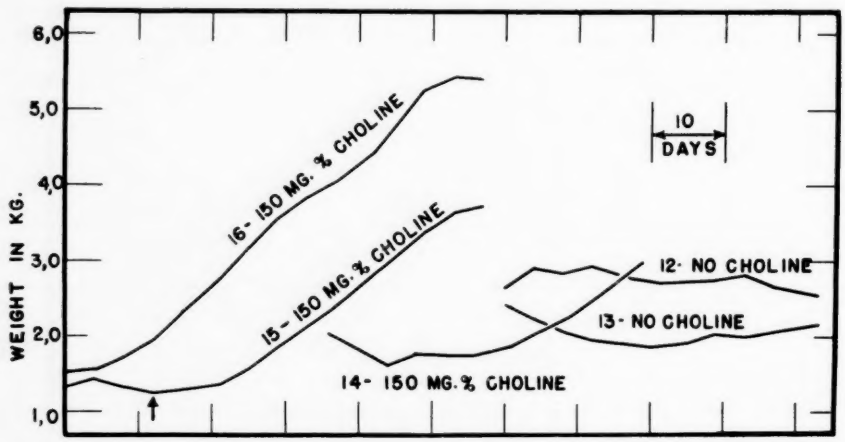

Fig. 3.—Growth curves of puppies of Experiment 3, Ration 3. Arrow indicates inception of supplement of 150 mg. choline chloride per 100 Gm. of ration.

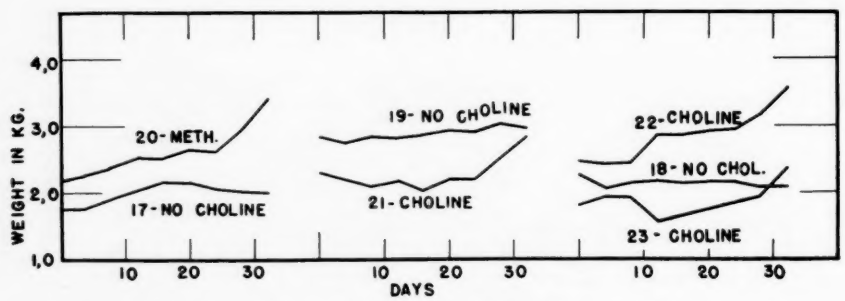

Fig. 4.—Growth curves of puppies of Experiment 4, Ration 3.

He was then given 0.5 Gm. choline chloride orally and then 150 mg. per cent were added to the ration. After a lag of ten days he began to grow and grew well thereafter. Dogs 12 and 13 received no choline and showed a net loss of weight over the experimental period of forty-three days. At this time they were sacrificed for tissue examination. Dog 14, a littermate of Dogs 12 and 13, received 150 mg. per cent of choline chloride. This dog failed to make any gain in weight until the twenty-eighth day. From this time on its weight and activity steadily improved until the forty-third day, when it was sacrificed for tissue examination.

The third litter placed on Ration 3 contained seven puppies (Experiment 4 and Fig. 4). Dogs 17, 18, and 19 were given the ration without additional supplements. The ration of Dog 20 was supplemented with 0.7 per cent dl-methionine, and that of Dogs 21, 22, and 23, with 150 mg. per cent choline

TABLE II

TERMINAL BLOOD AND TISSUE FINDINGS IN PUPPIES OF EXPERIMENTS 3 AND 4

DOG	SEX	RATION	SUPPLEMENT	DAYS ON EXPERIMENT	LIVER % $CHCl_3$ EXTRACT	BROMSULFALEIN TEST μG. DYE PER ML. PLASMA	PLASMA PHOSPHATASE μG. P LIBERATED		PROTHROMBIN TIME SEC.	HEMOGLOBIN GM. %	HEMATOCRIT %	RED BLOOD CELLS MILLION PER $MM.^3$
							8 HR.	24 HR.				
12	F	3	None	43	50.5	12	397	998	23.0	11.1	41.5	5.3
13	F	3	None	43	48.1	25	624	1,230	13.0	9.2	29.5	4.4
14	F	3	150 mg. % choline chloride	43	7.4	4	122	260	8.0	9.8	30.0	5.3
15	M	3	150 mg. % choline chloride	57	13.3	3	79	231	10.8	10.1	31.0	4.8
16	F	3	150 mg. % choline chloride	57	16.0	5	87	222	10.8	10.8	35.0	4.3
17	F	3	None	32	47.1	31	563	1,090	62.0	9.0	36.0	4.1
18	M	3	None	32	56.6	42	566	1,183	46.0	9.6	41.0	4.6
19	M	3	None	32	48.2	22	614	1,124	26.0	10.7	45.5	4.8
20	F	3	0.7% dl-methionine	32	11.9	10	82	245	13.7	10.6	39.0	5.8
21	M	3	150 mg. % choline chloride	32	19.1	4	137	349	10.5	10.1	37.0	5.1
22	F	3	150 mg. % choline chloride	32	14.1	4	112	300	12.0	11.0	40.0	7.0
23	M	3	150 mg. % choline chloride	32	12.3	5	143	399	8.8	9.3	30.0	4.8

chloride. Food consumption and growth in the animals receiving the supplemented rations were little better than in those on the basal ration for the first three weeks but showed improvement from this time until the thirty-second day, when all the puppies were sacrificed. Terminal hepatic dye clearance (bromsulfalein), plasma phosphatase, and prothrombin times were determined on all of the twelve dogs. These and other terminal findings are summarized in Table II.

In order to obtain information on the quantitative requirement of the growing pup for choline and on the biochemical nature of the deficiency, two more litters of puppies were placed on experiment. The first litter of seven puppies (Experiment 5, Fig. 5) was placed on Ration 4. In this ration, the liver extract was the same as that used in Ration 3, but the concentration was reduced to 2 per cent. Two of the dogs received no choline supplements, one received 5 mg. per 100 Gm. of ration, another 10 mg., two others 150 mg., and the last 0.7 per cent dl-methionine on the ration. Since the dogs did not eat the ration well, it was thought that more Liver Fraction "L" might be required, and they were all given a total of 11.5 Gm. per kilogram of body weight orally in the period from the seventeenth to twenty-first days. Striking improvement in the liver function tests of the two dogs with choline deficiencies occurred after this liver fraction feeding, and one of them (Dog 26) sacrificed at this time showed a relatively normal liver lipid content. Whether this liver repair was due to the 10 mg. of choline per kilogram of body weight obtained

TABLE

TERMINAL BLOOD AND TISSUE FINDINGS

DOG	SEX	RATION	SUPPLEMENT	DAYS ON EXPERIMENT	LIVER % $CHCl_3$ EXTRACT	LIVER CHOLESTEROL MG. PER GM. DRY	BROMSULFALEIN TEST µG. DYE PER ML. PLASMA	PLASMA PHOSPHATASE µG. P LIBERATED	
								8 HR.	24 HR.
24	M	4	150 mg. % choline chloride	41	20.5		7	105	331
25	F	4	150 mg. % choline chloride	43	13.1		6	85	239
26	F	4	None	21	19.5		12	86	236
27	M	4	None	41	27.5		24	534	1,220
28	F	4	10 mg. % choline chloride	41	27.9		24	353	931
29	M	4	5 mg. % choline chloride	43	30.4		47	572	1,159
30	F	4	0.7% dl-methionine	41	11.8		8	115	341
31	F	5	None	18	34.6	9.1	25		1,423
32	F	5	None	21	46.3	8.3	27		1,403
33	F	5	10 mg. % choline chloride	60	48.7	8.4	20	629	1,358
34	F	5	25 mg. % choline chloride	60	43.2	8.7	20	562	1,098
35	F	5	50 mg. % choline chloride	60	25.9	8.0	9	114	316
36	F	5	100 mg. % choline chloride	60	16.9	9.6	9	72	239

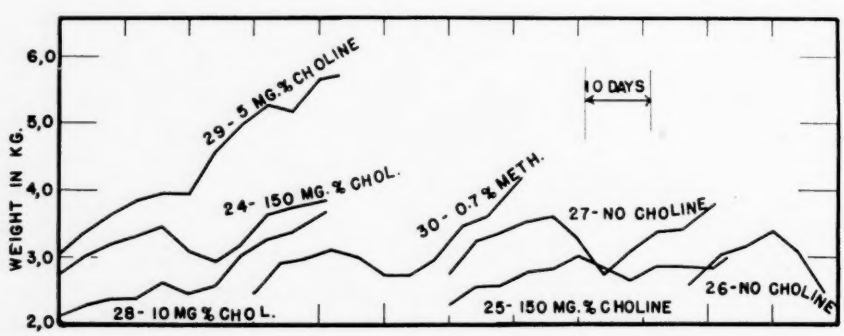

Fig. 5.—Growth curves of puppies of Experiment 5, Ration 4.

from the liver extract or to other lipotropic substances present in the liver extract cannot be stated. The liver feeding was stopped on the twenty-first day, however, and the experiment was continued until the fortieth day. At this time, liver function tests in the dogs receiving no choline and those receiving 5 mg. and 10 mg. per cent choline indicated considerable liver disease. All the dogs were sacrificed at this time. Terminal blood and tissue findings are given in Table III.

The failure of Ration 4 to provide good growth in all the control dogs when supplemented with 150 mg. per cent choline, coupled with the low plasma protein concentrations found in these animals, suggested that the ration was inadequate in protein. At this time the peanut oil meal became unavailable, and we were forced to obtain peanut protein from peanut flour. After ex-

III

IN PUPPIES IN EXPERIMENTS 5 AND 6

PROTHROMBIN TIME SEC.	PLASMA TOTAL CHOLESTEROL MG. %	PLASMA CHOLESTEROL ESTERS MG. %	BLOOD GLUCOSE MG. %	HEMOGLOBIN GM. %	HEMATOCRIT %	PLASMA PROTEINS GM. %	BLOOD CREATININE MG. %	MUSCLE "TOTAL" CREATINE MG. PER GM. FRESH	
								SKELETAL	HEART
11.5	97	67		11.5	37.5	3.9	1.10	3.50	
11.6	110	57		9.7	33.5	3.5	0.76	3.27	2.66
10.0				12.0	40.5	5.7	1.09	3.26	
13.2	60	30		11.8	39.0	4.2	0.78	3.35	
13.8	70	43		14.2	40.0	4.2	0.96	3.04	
18.0	83	22		11.5	39.0	3.6	0.78	3.48	2.52
10.0	90	60		11.6	32.5	4.8	1.14	3.48	
55.0	37	9		3.6	10.0	3.5	0.72	2.72	1.87
23.8	40	22	194	1.8	6.0	2.4	0.65	2.72	1.83
16.3	65	37	144	10.3	33.5	4.5	2.38	2.58	2.06
16.2	39	27	119	8.7	32.0	4.0	1.21	2.70	1.95
11.8	79	58	124	13.1	38.0	5.0	1.88	2.71	2.14
12.9	158	95	112	11.5	34.5	4.8	1.58	2.72	2.04

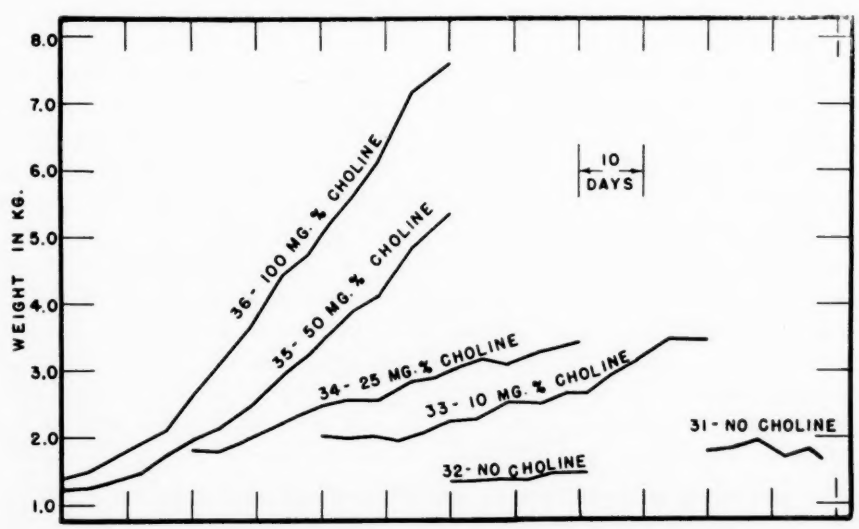

Fig. 6.—Growth curves of puppies of Experiment 6, Ration 5.

traction this peanut flour contains 64 per cent protein (N × 6.25). It was decided to include the extracted peanut flour at a level of 15 per cent and to raise the casein content of the ration from 7 to 10 per cent. This change was expected to increase the value of the protein in the ration and would at the same time decrease the choline content of the ration. The composition of this ration is shown in Table I (Ration 5). A litter of six puppies was placed on

this ration (Experiment 6, Fig. 6). Two received the basal ration only, and the other four received the basal ration supplemented with 10, 25, 50, and 100 mg. of choline chloride per 100 Gm., respectively. Growth in Dog 36, receiving 100 mg. per cent choline, was excellent; fair in Dog 35, receiving 50 mg. per cent choline; and poor in Dogs 33 and 34, receiving 10 and 25 mg. per cent choline, respectively. Dogs 31 and 32, receiving no choline supplement, died on the eighteenth and twenty-first days, respectively. The onset of this fatal deficiency was rather sudden but was preceded by loss of appetite and activity several days before death. The animals were markedly undernourished, and there was pallor of the viscera. The liver was yellow, and the thymus gland was considerably smaller than normal. These observations are consistent with our findings in all of the dogs deficient in choline in Experiments 3, 4, and 5. Gross pathologic examinations were made by Dr. Frank Dutra, of the Department of Legal Medicine, and samples for histologic examination were taken by him; they will be discussed in a later publication. The remaining dogs in Experiment 6 were sacrificed on the sixtieth day for examination. Blood and tissue findings in this litter of dogs are summarized in Table III.

METHODS

The total lipid analysis of all liver samples was performed in the following way: The livers were dried in a constant temperature oven at from 62 to 65° C., ground in a small mortar, and extracted in a Soxhlet extractor for thirteen hours with chloroform. The per cent of chloroform-extractable material is given in terms of dry weight of liver throughout and is an average of two separate analyses.

Liver cholesterol was determined by the Liebermann-Burchard color reaction developed from a suitable aliquot of the chloroform solution of the liver lipids. The color of the extracts was corrected for by reading in the spectrophotometer at 710 μ before adding the sulfuric acid. Although this color may have changed somewhat by the addition of the acid, the error is necessarily small since these colors adsorb very little at 710 μ.

Blood plasma cholesterol and cholesterol esters were determined by the method of Bloor[7] and Bloor and Knudson,[8] respectively, and adapted to the spectrophotometer. Blood hemoglobin was determined by the acid hematin color measured in the spectrophotometer at 380 μ. Blood sugar was determined by the micromethod of Folin and Svedberg[9] adapted to the spectrophotometer. Plasma proteins were determined by the plasma gravity method of Phillips and co-workers,[10] using the equation:[11]

(Plasma Specific Gravity—1.00687) 340.1 = Gm. plasma proteins per 100 c.c.

The plasma protein determinations were frequently checked by the Kjeldahl method and favorable agreement found.

Blood creatinine was determined by development of the alkaline picrate color in tungstate filtrates of blood and measuring the color in the spectrophotometer at 500 μ. Muscle "total" creatine was determined by autoclaving finely cut muscle tissue in 2N sulfuric acid for forty-five minutes. The alkaline picrate color was developed from a tungstate filtrate of this preparation and measured in the spectrophotometer.

The bromsulfalein test was modified from the usual procedure in the clinic to one more suitable for work with dogs. Seven milligrams of bromsulfalein per kilogram of body weight were injected intravenously instead of the usual 2 to 5 mg., and the plasma samples were obtained after an interval of eight minutes. Normal adults and puppies have an almost colorless plasma after this time. This test is based on the quantity of dye present in the plasma after a given time (eight minutes) rather than the usual procedure of establishing the time required for the plasma to be completely decolorized. In practice all samples were obtained within plus or minus twenty seconds of the eight-minute mark by stop watch. The average deviation of all samples from eight minutes was thirteen seconds. Twelve-minute samples were obtained from several of the severely deficient dogs. It is interesting that these all contained over 80 per cent of the amount of dye present at eight minutes. Only dye concentrations of the eight-minute samples are listed in the tables. The determination of bromsulfalein in the plasma consisted of adding 4.5 c.c. distilled water and 1.0 c.c. normal NaOH in this order to 0.5 c.c. of the plasma in a spectrophotometer tube. The colors were immediately read in the spectrophotometer at the absorption maximum of 580 μ. Since other substances in plasma absorb in highly varying amounts at this wave length, we found it necessary to use a "blank" for each dog made up with a sample of plasma taken before the dye injection.

The plasma phosphatase was determined by adding 1.0 c.c. of plasma from oxalated blood to 5 c.c. of 0.3 per cent sodium glycerophosphate adjusted to pH 7.6 with dilute HCl. Three tubes were used for each dog. One and one-half cubic centimeters of 10 per cent trichloracetic acid were added to the first tube immediately; 5 drops of chloroform were then added to each tube, and the tubes were incubated at about 38° C. After eight hours, 1½ c.c. of 10 per cent trichloracetic acid were added to Tube 2 and after twenty-four hours, to Tube 3. The tubes were then centrifuged and suitable aliquots removed for inorganic phosphate analysis. This was done colorimetrically by the Fiske and Subbarow method, using the spectrophotometer at a wave length of 660 μ. The zero hour phosphate was subtracted from the eight- and twenty-four-hour values in order to calculate the amount of inorganic phosphate liberated by the enzyme. No attempt is made to convert phosphorus values to enzyme units of any kind. Figures given are merely micrograms of phosphorus liberated by 1 c.c. of plasma in eight and twenty-four hours.

Prothrombin times were determined by rapid removal of jugular blood into a dry syringe and expressing 1 c.c. of this blood into a 9 mm. bore tube containing 0.2 c.c. of freshly prepared Winthrop's "Niphanoid" thromboplastin. The tube contents were mixed and the tube tipped back and forth until the clot formed. Two determinations were done with each dog. The method at best is crude and subject to limitations in interpretation, but after standardizing our technique, we were able to obtain excellent checks in control dogs. There was more variation in the deficient dogs. Prothrombin times are given in seconds from the time the 1 c.c. of blood came into contact with the thromboplastin. There is a few seconds' interval between the time the blood enters the syringe and is expressed into the tube, but our technique was uniformly rapid and this should not introduce a relative variable.

DISCUSSION

The failure of the control dogs receiving choline in Experiments 1 and 2 and the response of two of these animals to a liver fraction indicate that one or more substances essential for the nutrition of the dog were lacking on these rations. Since casein and methionine were ineffective in promoting growth in these aniamls, it was unlikely that amino acids were the sole deficiency factor. Improvement was afforded by using another liver extract (fraction "L") in Experiments 3, 4, 5, and 6. These results are surprising in view of past experience with purified rations for the dog. On purified rations supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline as sources of the vitamin B complex, fatal deficiencies were not observed over an experimental period of nine months. Only an occasional growth impairment or loss in weight[12] suggested that another factor was needed. Indeed an excellent ration for the production of pantothenic acid deficiency in the dog[13] contained no natural vitamin B complex source whatever. This was a highly purified ration supplemented only with synthetic B vitamins. Growth was excellent in the control dogs receiving pantothenic acid, and there was little reason to suspect the necessity of an additional factor. However, other investigators have observed fatal deficiency in dogs receiving rations of this type over extended periods of time.[14] Perhaps differences in the synthesis of nutrients by microorganisms of the gastrointestinal tract may account for these variations, although why synthesis might be different in the animals receiving the peanut meal ration is not known. It is also possible that the peanut meal exaggerates in some way the requirements for certain nutrients. Little can be said regarding the nature of the nutrient in Liver Fraction "L" essential in these experiments. It is probably not biotin, since the Fraction "L" is low in biotin and since a liver residue rich in biotin was found to be ineffective in preventing the death of Dogs 7 and 8 in Experiment 2. Fraction "L," however, is high in eluate factors essential for chicks and certain bacteria. The low plasma proteins in all the dogs of Experiment 5 (Table II) and the relatively poor growth of most of the control animals in Experiments 3, 4, and 5 (Figs. 3, 4, and 5) suggest, in addition, that the digestibility or quality of the peanut protein may be poor for the young pup. The data indicate that Rations 1 through 4 are not adequate for the weanling puppy, although choline deficiency may invariably be produced on them. However, Ration 5, which was used in Experiment 6, seems to meet all requirements, since excellent growth and higher plasma protein values were obtained in the control dog fed 100 mg. per cent choline, yet the ration was sufficiently deficient in choline to permit the development of a fatal deficiency in less than three weeks.

The growth and general condition of the methionine-fed dogs (20 and 30 in Experiments 4 and 5) indicate that this substance is effective in protecting the dog from choline deficiency. This is in accordance with the findings[15] in rats. Thus the deficiency appears to be primarily of labile methyl groups and not of choline per se. There is, however, a small amount of choline in the ration since the liver extract (Fraction "L") contains approximately 0.9 mg. per gram, and presumably there are small amounts of choline phosphatides in the cottonseed oil and perhaps protein-bound choline still unextracted from the

peanut meal. Thus there remains the possibility of a specific requirement for choline per se, but, if existent, it is probably small in relation to the amount of labile methyl required.

Experiment 6 gives the clearest indication of the quantitative requirement for choline, since excellent growth was observed in the ration supplemented with 100 mg. per cent choline, and since this ration (5) contains the least amount of choline. It seems clear that 100 mg. of choline per 100 Gm. of ration was entirely adequate for Dog 36, since the rate of growth was optimum for laboratory dogs, and that 10 and 25 mg. per 100 Gm. of ration were inadequate for Dogs 33 and 34. Dog 35 showed a slightly fatty liver and growth somewhat inferior to that of Dog 36. Thus 50 mg. per cent appears somewhat inadequate on this ration, although the experiments are limited.

Food consumption studies show that the growing pup consumes from 50 to 60 Gm. of this ration per kilogram of body weight per day. The choline requirement *on this ration* must therefore be no more than 60 mg. of choline chloride per kilogram of body weight per day. This requirement is interesting in view of the requirement of the depancreatized dog. The amounts of choline and methionine in the ration used by Best and co-workers[1] were considerable, since it contained almost 75 per cent lean meat. These animals required, in addition, 1 Gm. or more of choline per day in order to prevent the fatty degeneration of the liver. Dragstedt[16] also states that depancreatized dogs require "approximately 2.0 Gm. of choline a day over and above that present in the diet" to prevent the fatty metamorphosis of the liver. It should be noted that our experiments were all done with growing pups, and pups might be expected to require more choline than adult dogs. Reducing all these factors to a rough estimate, it would seem that a depancreatized dog might require over three times the amount of choline required by a normal dog. Considerable change in choline or labile methyl metabolism must therefore accompany pancreatectomy, and it is possible that other substances such as the "lipocaic" of Dragstedt,[16] which are lipotropic but not choline and which are contained in raw pancreas, merely operate to restore the prepancreatectomy choline metabolism.

The rapidity with which choline deficiency appears under these conditions is somewhat comparable to that observed in rats[17] and is a confirmation of the findings of Hough and associates.[4] These investigators used a peanut meal ration similar to that described in this paper except that it was much higher in fat. They observed striking differences in hepatic dye clearance (rose bengal) and in serum phosphatase after their puppies had been on the ration only fourteen days. It is unfortunate that their animals developed infection and died before completion of the experiment, since further observations on the adequacy of their ration for the control animals would be most interesting in veiw of our experience with this type of ration.

Fouts[3] reported a nutritional cirrhosis in dogs on a low choline ration which was isocalorically much higher in fat and lower in protein than the ration described in this paper. The protein was exclusively casein, so the methionine content was also greater than that of our rations, and liver fractions or other vitamin B complex sources were not used in most of the dogs. Adult dogs were used instead of puppies in most of the experiments, and the author states that there was a weight loss in all the dogs. The factors of age, lack of growth, and

higher methionine content of the ration point to a more chronic type of deficiency, as was actually the case. One group of dogs averaged 175 days survival on the ration.

The lipid content of the liver of the animals with choline deficiencies (Tables II and III) was extremely high and is comparable to that observed in the fatty infiltration of the liver of the puppy with pantothenic acid deficiency.[13] The control range of from 7.4 to 20.5 per cent and the deficient range of from 25.9 to 56.6 per cent (dry weight basis) are quite distinct and all liver, with one exception, has been confined to these ranges. The exception was in the case of Dog 26 with choline deficiency in which the liver contained 19.5 per cent lipid. This animal was, as mentioned previously, sacrificed after the feeding of liver extract (Experiment 5), and this may have exerted lipotropic action due to its content of choline or other substances.

The total cholesterol values of the liver obtained in Experiment 6 (Table II) indicate that in spite of the rise of total lipids in the liver during the course of choline deficiency, the cholesterol content of the liver is relatively unaffected.

The elevation of the plasma phosphatase in the deficient dogs is clearly seen from Tables II and III. That of the choline-fed control animals ranged from 72 to 143 μg. phosphorus liberated per cubic centimeter of plasma in eight hours and from 222 to 399 μg. phosphorus in twenty-four hours. Typical values for dogs receiving no choline are 614 μg. phosphorus in eight hours, and 1,124 μg. in twenty-four hours (Dog 19). In general, the increase in phosphatase activity is related to the degree of fatty infiltration of the liver.

There is also clearly demonstrated a marked impairment in the removal of bromsulfalein from the plasma of the deficient dogs. The plasma from dogs receiving choline contained from 3 to 10 μg. of bromsulfalein per cubic centimeter of plasma after eight minutes, whereas dogs receiving no choline had from 12 to 42 μg. of the dye per cubic centimeter of plasma. However, the results of the bromsulfalein tests are not as well correlated with the degree of fatty infiltration of the liver as are the phosphatase values.

The prothrombin time increases were less striking, on the whole, and appeared only in those animals having the most severe fatty infiltration of the liver. Since all control animals were confined to the range 8.0 to 13.7 seconds, this must be regarded as "normal" under these conditions. Deficient Dogs 13, 26, 27, 28, and 29 all had prothrombin times in this range, and of these only one had a liver lipid content over 31 per cent. Much higher prothrombin time readings were observed in Dogs 12, 17, 18, 19, 31, and 32 (twenty-three to sixty-two seconds), and these animals had an average liver lipid content of 47.2 per cent.

The total cholesterol and cholesterol esters of the plasma in the control animals ranged from 90 to 158 and from 57 to 95 mg. per cent, respectively. The deficient animals ranged from 40 to 83 and from 9 to 58 mg. per cent, respectively. The average values for the control dogs are 114 mg. per cent total cholesterol and 70 mg. per cent cholesterol esters, while those for the deficient are 59 and 31 mg. per cent, respectively. Both cholesterol and cholesterol esters are therefore markedly reduced in the plasma of the animals deficient in choline, but the results indicate little correlation between the degree of fatty infiltration of the liver and the amount of lowering of either of the cholesterol components.

The changes in plasma phosphatase, cholesterol, and cholesterol ester values, bromsulfalein elimination, and prothrombin time are all commonly associated with liver disease. Most likely they do not reflect a primary biochemical effect of choline deficiency but rather a secondary effect incident to the fatty infiltration of the liver. The fall in hematocrit, hemoglobin, and plasma proteins observed in Dogs 31 and 32 and the rise in blood sugar in Dog 32 are doubtless other manifestations of this liver dysfunction. Evidently when a longer time is required for the liver to become infiltrated with fat, as in the case of Dogs 27, 28, 29, 33, and 34, the animal is able to maintain more normal concentrations of these substances.

The colloidal gold test as described by Gray[18] was also applied to the sera of these dogs but revealed no differences whatever in the flocculating power of the sera between the dogs with severe liver impairment and those with normal liver findings. This was true in all ranges of sensitivity of the colloidal gold solution. As the colloidal gold test depends on the presence in the serum of increased amounts of gamma or beta globulin,[19] it suggests that such increases do not occur in the pup with choline deficiency.

The dependence of creatine synthesis on the methyl "pool"[20] led us to investigate blood creatinine and muscle "total" creatine concentrations to see if there were any serious impairment in creatine formation. The skeletal muscle used throughout these studies was the pectoralis major. The two litters of dogs studied (Dogs 24 through 36, Table III) gave different "total" creatine concentration ranges. We are unable to explain these differences, but it can be seen from Table III that in either single experiment "total" creatine in both skeletal and heart muscle was not lowered in severe deficiency. This is in accordance with the findings of Almquist and co-workers[21] in chicks and of Roberts and Eckstein[22] in rats. There were no differences in the per cent dry weight of muscle tissue from dogs receiving choline and those receiving no choline, so the relationship of the creatine values holds in terms of both wet and dry muscle. The blood creatinine values are highly variable and difficult to correlate in any way with the state of nutrition of the animals. Whether or not total creatine excretion studies would indicate a decreased synthesis of creatine in the pups deficient in choline remains to be investigated. It is interesting that a tissue as rich in creatine as skeletal muscle can maintain a normal creatine content in the face of a severe methyl deficiency.

SUMMARY

The development of a ration for the production of acute choline deficiency in weanling puppies is described. Fatal deficiency in choline may be produced in less than three weeks and is characterized by severe fatty infiltration of the liver. Seven-tenths per cent dl-methionine or 0.1 per cent choline chloride supplemented to the ration apparently renders it adequate in all respects. In choline deficiency there is a rise in blood plasma phosphatase, an impairment in bromsulfalein elimination, and a fall in blood plasma cholesterol and cholesterol esters. In severe choline deficiency there is, in addition, an increase in prothrombin time and a decrease in blood hemoglobin, hematocrit, and plasma proteins. "Total" creatine in both heart and pectoralis major muscles is not lowered in choline deficiency, nor is there a consistent lowering of blood creatinine.

The total cholesterol concentration of the liver is unchanged, although total lipids are increased from three to four times.

The choline requirement of the growing puppy on this ration is probably not more than 100 mg. per 100 Gm. of ration or 50 mg. per kilogram of body weight per day.

We are indebted to Merck & Co., Inc., Rahway, N. J., for generous supplies of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, choline, and methionine; to The Wilson Laboratories, Chicago, Ill., for liver extracts; to Abbott Laboratories, North Chicago, Ill., for liver extracts; and to Hynson, Westcott, and Dunning, Inc., Baltimore, Md., for bromsulfalein.

The authors wish to thank Mr. Fred Snell and Mr. Jose Sarraga-Audinot for their assistance in this work.

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LYSOLECITHIN AND THE ANTIHEMOLYTIC VALUE OF THE BLOOD

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Bergenhem and Fåhraeus[1] have found that when mammalian blood is incubated the erythrocytes tend to become spherical and their sedimentation velocity greatly reduced. Production of lysolecithin was believed to be responsible for these changes, and increased quantities of lysolecithin could be extracted from incubated serum, as compared with fresh serum. If the blood or serum were agitated during incubation, the changes did not take place. It was suggested by these authors that lysolecithin is formed by an enzymatic process in the relatively stagnant blood of the spleen, and that in the peripheral circulation the rapid movement of the blood prevents the formation of lysolecithin.

Singer[2] described an extraction method for the quantitative estimation of lysolecithin in serum; he confirmed the observation that lysolecithin increases on incubation of unmoved blood and that shaking inhibits the process. An increased production of lysolecithin in the stagnant blood from the splenic vein, and from varicose veins, was indicated. In a further investigation, Singer, Miller, and Dameshek[3] found that splenectomy caused definite alterations in lysolecithin metabolism. Gripwall,[4] using Singer's method, has confirmed most of these findings.

Nevertheless, it has not been conclusively proved that lysolecithin plays an important role in normal blood destruction, as Singer[2] admits. Furthermore, Gillespie[5] has recently shown that the changes that take place in the erythrocytes of incubated blood are not the same as the changes produced by lysolecithin. Finally, it may be pointed out that Bergenhem and Fåhraeus[1] based their conclusions mainly upon measurement of sedimentation velocities, a method clearly not specific for lysolecithin. The reliability of Singer's extraction method has not been demonstrated, and in the present investigation it has been found that this method does not give quantitative recovery of added lysolecithin. In the absence of a specific quantitative method for determining lysolecithin in the blood, it was decided to adopt a direct titration method, which gives a measure of the net antihemolytic value of whole blood, or of serum, in terms of lysolecithin.

METHOD

The method employed is based upon the assumption that changes in the concentration of lysins, or of antihemolytic substances, in the blood will be reflected in the amount of lysolecithin required to bring about hemolysis of that blood under stated conditions. The procedure, a modification of that of Collier and Mack,[6] consists of a determination of the number of milligrams of added lysolecithin which will bring about 50 per cent hemolysis of 1 c.c. of blood, or its equivalent, in 60 seconds at room temperature. This number is termed the

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Received for publication, June 16, 1944.

antihemolytic value. (The temperature coefficient of lysolecithin hemolysis was found to be very low—only 1.05 for the range 20 to 30° C.)

Apparatus.—A Lumetron Model 401 photoelectric colorimeter, with ½-inch tubes and red filter No. B660, was used for following hemolysis by measuring the opacity of the cell suspension as described by Wilbur and Collier.[8]

The hemolysis blank was negligible, and cell suspensions were found to obey Beer's Law, i.e., extinction or optical density (– log transmission) was proportional to cell concentration.*

Material.—The lysolecithin was the snake venom preparation of Collier and Allen[7]; although not pure, it provided a reproducible standard. It was dissolved in absolute methanol at a dilution of 1:1,000 for whole blood or serum and 1:4,000 for erythrocytes alone.

Buffered saline (4 volumes of 0.95 per cent NaCl plus 1 volume of M/8 sodium phosphate buffer, pH 7.4) was used for diluting the blood samples. Redistilled water was used for preparing solutions and for rinsing glassware.

Procedure.—For whole blood (anticoagulant 1:10,000 heparin†) 20 μl. of the sample were diluted to 5 c.c. in the buffered saline. The suspension was stirred with a bulb pipette and at zero time the lysolecithin was added (about 50 to 80 μl. of 1:1,000 solution from a pipette graduated in 1/1,000 c.c.). Following immediate stirring the initial optical density was quickly noted and the colorimeter dial was set at 50 per cent of this reading. The time required for the galvanometer to read zero again was measured with a stopwatch, thus giving the time for 50 per cent hemolysis.

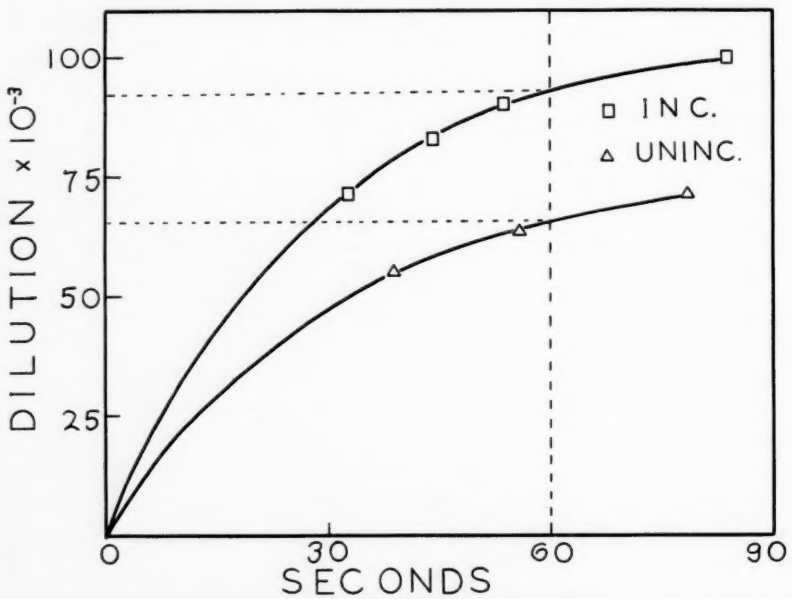

Fig. 1.—Lysolecithin time-dilution curves for human blood. Ordinates: lysolecithin dilution × 10^{-3} (e.g., 50 indicates a dilution of 1:50,000).

Several measurements were obtained for each sample, with hemolysis times somewhat less than or more than 60 seconds. From these values a conventional

*If a photoelectric colorimeter is not available, a visual colorimeter may be used for measuring opacity. The cell suspension is placed in one cup and a standard of opacity in the other. Under these conditions a longer time than 60 seconds for the end point is desirable.

†Connaught Laboratories.

time-dilution curve was plotted, with time of hemolysis as abscissae and lysolecithin dilution as ordinates. (For example, 50μl. of 1:1,000 lysolecithin in 5 c.c. = 1:100,000. Dilution = 100,000.) Typical time-dilution curves are given in Fig. 1.

From such a curve the dilution of lysolecithin required to bring about 50 per cent hemolysis in exactly 60 seconds is obtained by interpolation. Usually three points are sufficient and the precision of the method is about 2 to 3 per cent. From this dilution the antihemolytic value is calculated. For example, if the dilution corresponding to 60 seconds is 100,000, this is equivalent to 50 μg of lysolecithin per 5 c.c. of 1:250 blood, or 2.5 mg. of lysolecithin per 1 c.c. of whole blood. The antihemolytic value is therefore 2.5.

For erythrocytes alone the cells from 1 c.c. of blood were washed three times with the buffered saline and made up to 250 c.c. To 5 c.c. of this 1:250 suspenpension in the colorimeter was added 1:4,000 lysolecithin (about 50 to 100 μl.), and the dilution required for 50 per cent hemolysis in 60 seconds was determined as before.

The antihemolytic value of plasma was obtained by deducting the value for the cells from that of whole blood. For serum, 20 μl. were added to 5 c.c. of 1:250 cells of known antihemolytic value and the value for the cells alone was subtracted from that of the mixture. Type 0 cells were used for measuring a series of human untyped sera.

The antihemolytic value of blood reconstituted from washed cells and plasma was found to be identical with that of the whole blood, indicating that the use of washed cells is justified and that the antihemolytic value of plasma and of cells is additive. The sphering of cells induced by lysolecithin was not reversed by repeated washing.

The ratio of cells to plasma was obtained from hematocrit readings. In the case of incubated blood, the cells were so swollen that constant readings could not be made, and therefore plasma values could not be obtained directly.

When the Singer extraction method was applied to serum, the hemolysis readings were made photoelectrically in 1 c.c. microtubes, and the amount of lysolecithin in the extracts was determined by comparison with a hemolysis curve using standard lysolecithin and the same cells.

All incubations of blood or serum were carried out under sterile conditions.

RESULTS

Recovery of Lysolecithin by Singer's Method.—The Singer[2] extraction method was applied to both rabbit and human serum and the apparent increase in lysolecithin on incubation was confirmed. However, addition of lysolecithin to serum indicated that the recovery by this method was very low. When 0.20 mg. of lysolecithin was added to 10 c.c. of rabbit serum, none was recovered; addition of 10 mg. resulted in recovery of only 0.093 mg. Injection of 20 mg. of lysolecithin into a rabbit weighing 2.4 kg. caused no sphering of the cells and there was no increased recovery from the serum. This low recovery of added lysolecithin has been found to be due, in part, to incompleteness of the ether precipitation, apparent extraction of antihemolytic substances along with lysolecithin, and loss of activity of lysolecithin in saline solution.[8]

Finally, 10 mg. of lysolecithin were added to each 10 c.c. sample of human serum, incubated and unincubated; an apparent increase of 0.038 mg. per cubic centimeter of serum was found (Table III). This procedure is not economical enough for routine use, but it probably indicates the order of magnitude of the increase in lysolecithin on incubation.

Incubation and the Antihemolytic Value of Blood.—The antihemolytic value of blood or serum should be lowered on incubation if the concentration of lysolecithin increases, other factors remaining unchanged. Table I indicates that when rabbit serum was incubated twenty-one hours at 37° C. there was a decrease in the antihemolytic value of much greater magnitude than could be accounted for by the increase in lysolecithin as determined by Singer's method. Table IV summarizes the results of experiments on whole blood, cells, and serum of three species, rabbits, dogs, and human beings, showing that the antihemolytic value is always significantly decreased by incubation. The influence of various factors upon this change in the antihemolytic value during incubation is described below.

TABLE I

INCUBATION AND THE ANTIHEMOLYTIC VALUE OF RABBIT SERUM

	LYSOLECITHIN (MG. PER C.C.)		
	UNINCUBATED	INCUBATED	DIFFERENCE
Singer's method	0.0005	0.0016	0.0011
Antihemolytic value	2.92	1.96	0.96

That the change in the antihemolytic value of the erythrocytes is due in part to an alteration in the plasma is indicated by the following procedure. Washed cells from 1 c.c. of rabbit blood were mixed with 2 c.c. of incubated serum, and another aliquot of cells was mixed with 2 c.c. of the same serum refrigerated. After two hours the cells were washed again and their antihemolytic value was measured. The values were 0.50 and 0.67, respectively. Assuming that lysolecithin is adsorbed by erythrocytes from incubated serum,[1] this corresponds to an adsorption of 0.085 mg. of lysolecithin from 1 c.c. of incubated serum.

The change in the antihemolytic value with *time* of incubation at 37° C. was measured on human serum from two individuals and the results are plotted in Fig. 2. Control values show that some change took place even at refrigerator temperature and that there was a further marked decrease in the antihemolytic value when these controls were finally heated for thirty minutes at 60 to 63° C. The maximum change took place in about twenty-four hours at 37° C. and subsequent incubations were carried out for this length of time.

To determine the effect of *temperature* upon the change in the antihemolytic value of rabbit serum, samples of the same serum were heated for eight hours at various temperatures. The results of these experiments are given in Fig. 3. The serum at 62° C. was cloudy, indicating incipient denaturation. No temperature optimum was observed, and it was repeatedly found that heating serum between 60 and 70° C. resulted in a very marked decrease in the antihemolytic value.

The effect of *H-ion concentration* was determined, using both human and rabbit serum. The pH was adjusted by addition of NaOH or H_3PO_4 to the test tubes before sterilization, and the serum was incubated twenty-four hours

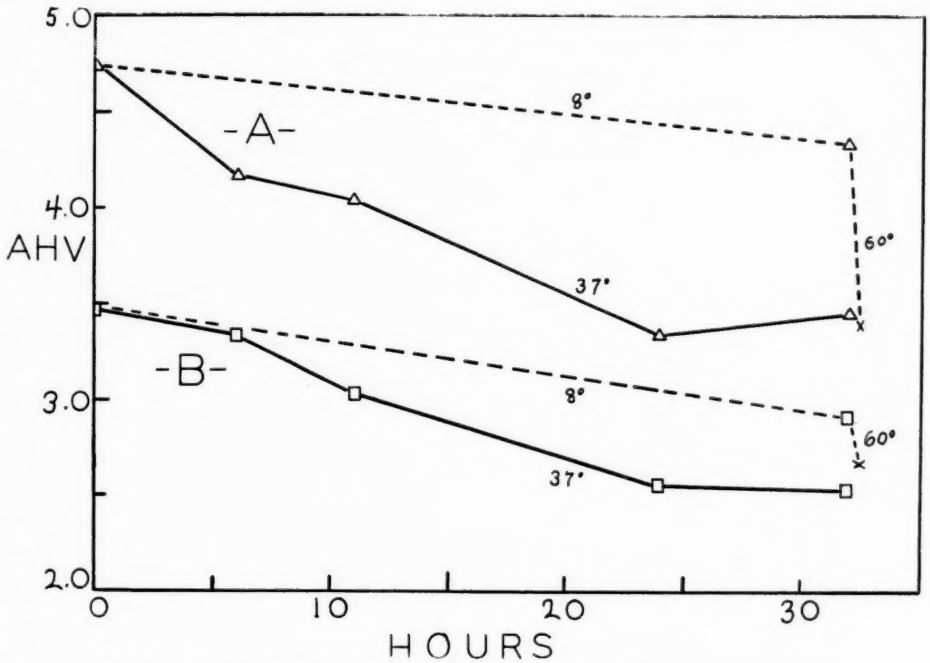

Fig. 2.—Change in the antihemolytic value on incubation of human serum.

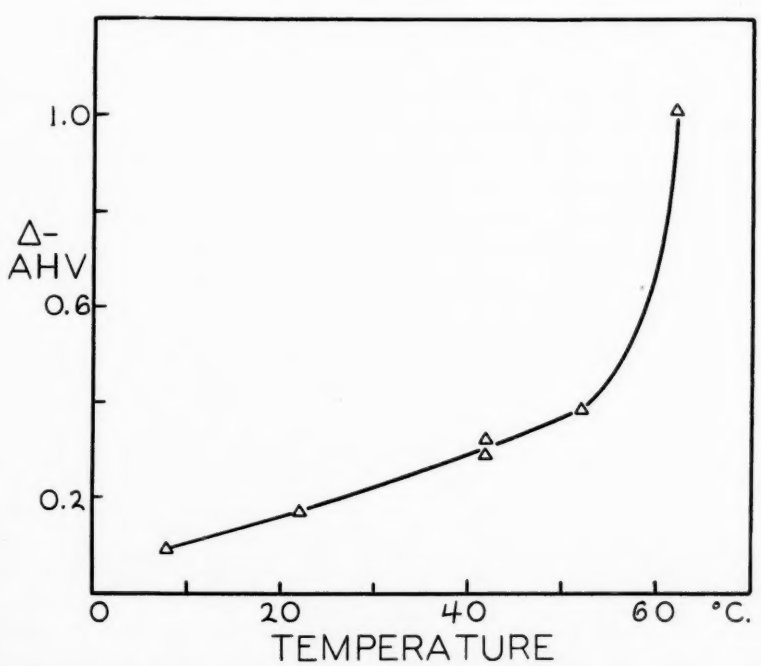

Fig. 3.—The effect of temperature on change in the antihemolytic value of rabbit serum. Ordinates: Δ – antihemolytic value = decrease in the antihemolytic value on incubation.

at 37° C. (The tubes must be stoppered with rubber stoppers; cotton plugs permit loss of CO_2, resulting in an increase of pH.) The results obtained with one human serum and two rabbit sera are given in Fig. 4. It is apparent that there is an optimum at about pH 7.5.

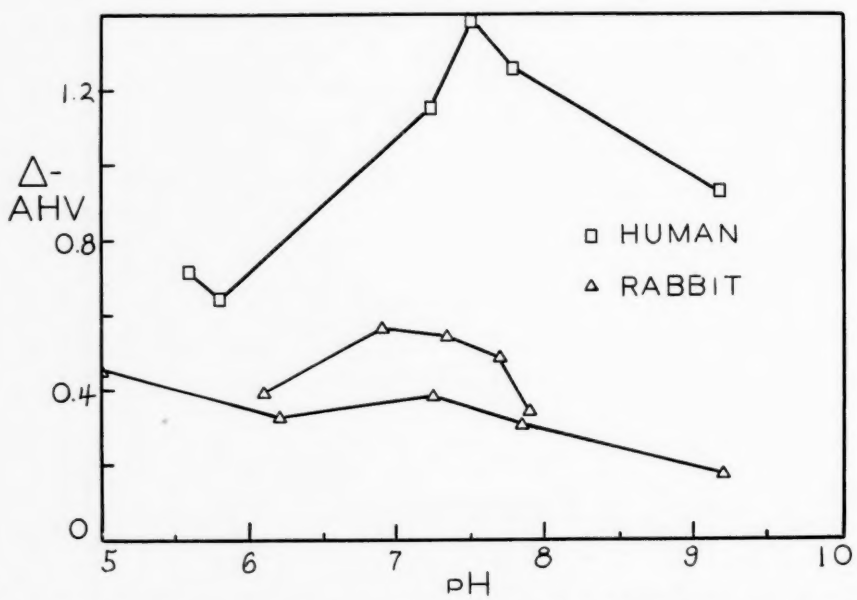

Fig. 4.—The effect of pH on change in the antihemolytic value of incubated serum. Ordinates: Δ – antihemolytic value = decrease in the antihemolytic value on incubation.

Shaking of serum or whole blood was carried out in L-shaped Pyrex rocker tubes, immersed in a constant temperature bath and rocked at fifteen oscillations per minute through an angle of 30 degrees. Control samples were simultaneously incubated at rest. The results of these experiments are summarized in Table II, from which it is seen that in four of six experiments rocking slightly diminished the change in the antihemolytic value.

TABLE II

THE EFFECT OF ROCKING ON THE ANTIHEMOLYTIC VALUE OF WHOLE BLOOD AND SERUM

	TEMP. (°C.)	DECREASE IN ANTIHEMOLYTIC VALUE		RATIO A/B
		(A) ROCKED	(B) AT REST	
Rabbit serum	20	0.40	0.40	1.00
Rabbit serum	37	0.36	0.44	0.82
Human whole blood	37	0.62	0.70	0.88
Human whole blood	37	0.54	0.67	0.80
Human whole blood	37	0.63	0.58	1.09
Human whole blood	37	0.70	0.73	0.96

Oxygen tension might be thought to have an effect on the changes taking place in stagnant blood, but experiments showed that reduction of the oxygen tension of whole blood had no marked effect on the decrease in the antihemolytic value. Samples of rabbit blood were evacuated, equilibrated with oxygen or nitrogen, and then incubated. It was found that the cells incubated under

N_2 were slightly more fragile and that a greater degree of spontaneous hemolysis had taken place. But the antihemolytic value of the whole blood showed no significant difference.

Various *enzyme inhibitors* were tested, but none of the following was found to have any effect on the change in the antihemolytic value of rabbit serum incubated twenty-four hours at 37° C.:

Quinine sulfate	1:5,000
Eserine sulfate	1:10,000
KCN	1:100,000
Tri-o-cresyl phosphate[9]	Saturated

Sodium fluoride, M/2,000, apparently accelerated the change in the antihemolytic value, but this may have been due to its hemolytic effect upon the erythrocytes. For this reason, fluoride is not a suitable anticoagulant for the measurement of the antihemolytic value. The oxalate mixture of Heller and Paul,[10] used as an anticoagulant, gave results similar to those obtained with heparin, except that the cells were somewhat increased in fragility, even when kept in the refrigerator.

Chemical Changes in Incubated Serum.—The antihemolytic value of blood plasma is a resultant of various hemolytic and antihemolytic factors. Among the lytic substances present in plasma may be lysolecithin, bile salts, and free fatty acid.[11] According to Tsai and Lee,[12] free cholesterol is the chief antihemolytic agent. In order to assess the magnitude of the changes of some of these constituents, human serum was incubated and the following determinations were carried out. The Singer extraction method for lysolecithin was applied following addition of 10 mg. of lysolecithin to each 10 cc. of incubated and unincubated serum. Free cholesterol (i.e., digitonin-precipitable) was precipitated by Sperry's[13] modification of the Schoenheimer-Sperry method, and the color was developed by the procedure of Rose, Schattner, and Exton,[14] a method that gives a relatively intense and stable color. Free fatty acid was determined by the method of Freeman and Johnson,[15] with electrometric titration in methanolic solution. The results of these analyses are given in Table III.

TABLE III

CHANGES IN CONSTITUENTS OF HUMAN SERUM ON INCUBATION

VALUES PER C.C. SERUM	UNINCUBATED	INCUBATED	DIFFERENCE	
			MG.	MICROEQUIV.
Antihemolytic value, as lysolecithin (mg.)	3.15	2.36	0.79	1.49*
Lysolecithin by Singer modified (mg.)	0.012	0.050	0.038	0.07*
Free cholesterol (mg.)	0.70	0.37	−0.33	−0.85
Free fatty acid (microequiv.)	2.20	1.45		−0.75

*Calculated as stearic lysolecithin.

It appears that the decrease in the antihemolytic value on incubation cannot be accounted for by the increase in extractable lysolecithin, and there is no increase in free fatty acid. The decrease in free cholesterol probably accounts for a considerable portion of the change in the antihemolytic value.

It was thought that chromatographic analysis might be applied to the separation of the serum lipids and to the precise determination of lysolecithin.

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Preliminary experiments have indicated that lysolecithin is strongly adsorbed by alumina from CCl_4 or $CHCl_3$ solution and that it may be eluted with alcohol. Free cholesterol can be removed from the lipid extract by the method of Trappe.[16] But when serum extracts were put through appropriate adsorption-elution procedures the recovery of added lysolecithin was still less than 1 per cent.

Summary of Antihemolytic Value Determinations.—Numerous antihemolytic value determinations have been carried out on the blood of rabbits, dogs, and human beings and the results have been summarized in Table IV. It is recommended, however, that each laboratory determine its own normal values rather than accept these figures as of absolute significance. (The antihemolytic value of blood specimens should be determined as soon as possible, as it decreases rapidly at room temperature and even in the refrigerator.)

TABLE IV

SUMMARY OF ANTIHEMOLYTIC VALUE DETERMINATIONS
(MEAN VALUES ± STANDARD ERRORS)

	NO. OF SAMPLES	UNINCUBATED	INCUBATED	DIFFERENCE
Rabbit whole blood	4	2.45 ± 0.09	1.83 ± 0.12	0.62 ± 0.07
Rabbit cells	4	0.72 ± 0.07	0.52 ± 0.04	0.20 ± 0.03
Rabbit serum	13	2.20 ± 0.11	1.81 ± 0.11	0.39 ± 0.03
Dog whole blood	2	3.31 ± 0.41	1.76 ± 0.05	1.55 ± 0.35
Dog cells	1	0.80	0.45	0.35
Dog serum	2	2.59 ± 0.25	2.32 ± 0.13	0.27 ± 0.12
Human whole blood	7	3.20 ± 0.12	2.30 ± 0.08	0.90 ± 0.06
Human cells	7	0.68 ± 0.01	0.49 ± 0.01	0.19 ± 0.01
Human serum	4	3.97 ± 0.27	2.90 ± 0.16	1.07 ± 0.12
Human serum*	24	3.85 ± 0.08		

*From normal blood donors.

The series of human sera was obtained from normal blood donors. There was no significant difference between the averages for nine males and for fifteen females. Three of the sera were slightly lipemic and gave rather high values; one very lipemic specimen, with an antihemolytic value of 6.0, was not included in the average.

Saponin may also be used for antihemolytic value determinations but the distribution between cells and plasma is different from that obtained with lysolecithin. When saponin is used, the cells make up about two-thirds of the antihemolytic value of whole blood, while the ratio with lysolecithin is about one-fourth. With saponin the change in the antihemolytic value on incubation is relatively small: a sample of human blood gave the values 13.9 and 13.2 before and after incubation.

DISCUSSION

In confirmation of the findings of Bergenhem and Fåhraeus[1] and of Singer[2] it has been found that the apparent lysolecithin content of serum increases on incubation. Singer's method gives, however, less than 1 per cent recovery of added lysolecithin and attempts to achieve quantitative recovery have been unsuccessful. (It is possible that the lysolecithin formed by snake venom is not identical with that in the serum. When sufficient serum lysolecithin is available, its recovery by Singer's method will be tested.) Because of the

inadequacy of this method and because of the possibility that blood may contain various hemolytic and antihemolytic substances, a direct titration of whole blood with lysolecithin is proposed and the result is expressed as the antihemolytic value.

The capacity of the blood to withstand the effects of lysins obviously depends upon the resistance of both cells and plasma. Therefore any measure of this resistance to hemolysis must include both components. A quantitative measure of the total hemolytic and antihemolytic factors has been given in terms of lysolecithin. (Other lysins could be used but lysolecithin was chosen because it is known to be present in the blood. It cannot be assumed, of course, that the various lytic substances in the blood act in additive fashion, but the antihemolytic value in terms of lysolecithin is suggested as an approximation to the resistance of the blood to lysis by various agents in vivo.) The present method, like those mentioned above, fails to give an accurate measure of the amount of lysolecithin in blood, so that the question of its physiologic significance must still remain open.*

The antihemolytic value of blood or of serum has been found to decrease markedly on incubation. When whole blood is analyzed both plasma and cells show a decrease in the antihemolytic value. At least a portion of the change in the cells is due to changes in the plasma. However, as Gillespie[5] has demonstrated, the volume and fragility changes that take place in the cells of incubated blood are not the same as those caused by lysolecithin.

Bergenhem and Fåhraeus[1] suggested that lysolecithin was produced in incubated blood through the action of a lecinthinase, similar to that of snake venom.[19] This enzyme in the blood was apparently completely inhibited by quinine, by shaking, or by heating to 52° C. The factors controlling the change in the antihemolytic value are evidently not identical with those that affect the sedimentation velocity, as shaking, lipase inhibitors, or heating did not inhibit the change. Furthermore, high temperatures accelerated rather than inhibited the change in the antihemolytic value, which fact suggests that the process is primarily nonenzymatic. The effect of pH is different from that observed by Bergenhem and Fåhraeus and more closely resembles the effect observed by Hughes[17] with snake venom acting on lecithin films. Denaturation of serum protein or of lipoprotein complexes may be involved in the alteration in the antihemolytic value: higher temperatures and lower pH values, causing cloudiness of the serum, resulted in a marked lowering of the antihemolytic value (Figs. 3 and 4). On the other hand, addition of rattlesnake venom as a source of enzyme resulted in a decrease in the antihemolytic value greater than that brought about by incubation alone.

It is probable that when blood is withdrawn from the circulation, both enzymatic and nonenzymatic changes are initiated. These take place even at refrigerator temperatures and are accelerated at higher temperatures. The decrease in the antihemolytic value cannot be accounted for by the apparent increase in extractable lysolecithin. Johnson[11] has discussed the lytic action

*The values in Table III, together with the data on the decrease in the antihemolytic value of cells mixed with incubated serum, suggest that the increase in lysolecithin on incubation of serum is of the order of magnitude of 0.05 to 0.10 mg. per cubic centimeter. A concentration of 0.25 mg. per cubic centimeter was necessary for sphering of the erythrocytes of rabbit whole blood in vitro.

of free fatty acids, but these decrease on incubation, possibly because of esterification with cholesterol. Furthermore, lipemic human serum has a somewhat elevated antihemolytic value rather than a lowered value. A great part of the decrease in the antihemolytic value of incubated serum may be due to the reduction in free cholesterol, a phenomenon which was described by Sperry and Stoyanoff.[18] If it is assumed that free cholesterol inactivates lysolecithin in molecular proportion,[19] more than one-half of the change in the antihemolytic value of human serum can be accounted for in this way (Table III).

The experiments on the antihemolytic value of blood offer no support for the "stagnation" hypothesis of normal hemolysis, since neither shaking nor reduction of oxygen tension had any marked effect on the antihemolytic value. Certain mechanisms must operate to maintain the antihemolytic value of the blood at a fairly constant level in vivo, but these evidently fail to operate in vitro. The practical significance of the antihemolytic value has not as yet been demonstrated, but it is planned to investigate the effect of various physiologic and pathologic conditions on the antihemolytic value of blood.

SUMMARY

1. It has been confirmed that extractable lysolecithin increases on incubation of serum. The Singer extraction method gives, however, less than 1 per cent recovery of added lysolecithin.

2. In the absence of a specific method for lysolecithin, the antihemolytic value of whole blood, erythrocytes, and serum has been determined by titration with lysolecithin. The antihemolytic value is defined as the number of milligrams of lysolecithin required to bring about 50 per cent hemolysis of 1 c.c. of blood, or its equivalent, in one minute at room temperature. Average values are presented for antihemolytic value determinations on blood of rabbits, dogs, and human beings.

3. The antihemolytic value of whole blood or serum decreases markedly on incubation. A rise in temperature accelerates the change, with no evidence of a temperature optimum. The pH optimum is at about pH 7.5.

4. The change in the antihemolytic value of incubated serum is not markedly affected by agitation, reduction in oxygen tension, or lipase inhibitors.

5. This change in the antihemolytic value may be partly due to the decrease in free cholesterol on incubation. It is probably the resultant of various changes in the serum or plasma proteins and lipids. It is not known whether this phenomenon has any physiologic significance.

We wish to acknowledge the kind cooperation of the staff of the Halifax Blood Donors' Clinic in obtaining small samples of human serum.

NOTE.—Attention should be drawn to a recent paper which appeared after this paper had been submitted for publication: Ponder, E.: The Kinetics of in vivo Hemolytic Systems, J. Gen. Physiol. **27**: 483, 1944.

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THE EFFECT OF HUMIDITY AND TEMPERATURE ON OXYGEN TOXICITY

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INTRODUCTION

Toxicity from oxygen has been repeatedly demonstrated.[1-6] The toxicity is dependent on the concentration of oxygen present and varies with the species.[2, 6, 7] In human beings concentrations as low as 70 per cent for a time as short as six days have been reported to cause oxygen toxicity.[8] In some species of animals, such as the rat, it also varies with age.[9, 10] In other species, such as the dog, no such age difference was found.[11] There is also evidence that oxygen toxicity is increased by increasing the external temperature. Thus Campbell showed that rats taken to six atmospheres of oxygen and slowly decompressed had a higher survival rate at 24° C. than at 33° C.[12] Turtles brought to mammalian temperature (37.5° C.) behaved like mammals, whereas at 23 to 26° C. they showed no symptoms.[3] The work of Paine and others showed that a dog whose external environment went to 90° F. did not survive as long as the other dogs which were maintained at a lower temperature.[11] The humidity has been recorded or kept constant in several studies on oxygen toxicity,[6, 7, 9-11, 13] but there seems to be a dearth of comparisons on the effect of humidity. It was our object, therefore, to study further the effects of temperature and humidity on oxygen toxicity.

EXPERIMENTAL PROCEDURE

A diagram of the apparatus used may be seen in Fig. 1. The chamber consisted of a 5-gallon glass jar with inlet and outlet tubes. The inlet tube was connected with two bottles which contained either water or calcium chloride, depending on the humidity desired. These connected directly with the oxygen tank or the outside air. Rats were placed in a wire cage within the chamber, four rats to a chamber (two on each side), at humidities 30 per cent and 90 per cent or higher. Two rats were used per chamber for humidities below 20 per cent. For the 30 per cent humidity, calcium chloride was placed in the stand holding the cage. For the lowest humidity it was necessary to surround the cage with a screen containing calcium chloride in addition to that in the stand. For humidity near saturation, water was placed about 1 cm. deep in the bottom of the chamber. A thermometer with a relative humidity measuring device was attached to the rat cage. These instruments were checked against wet and dry bulb thermometers and found to be accurate from about 10 to 40 per cent humidity. The high humidity experiments were

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Received for publication, July 27, 1944.

checked at intervals with the wet and dry bulb thermometers and also by Harger's glass syringe apparatus for determining moisture in air.[14]

Rats were selected as experimental animals because of their well-known resistance to oxygen poisoning.[9, 10, 15] Male rats were used throughout. The rats varied in weight from 69 to 111 Gm.; the average weight for the various groups varied from 79 to 91 Gm. The temperatures used for the experiment were 90 and 80° F., with variations of plus or minus 2 degrees. The humidities studied were a low humidity between 10 and 20 per cent, an intermediate humidity averaging about 30 per cent with maximum variations from 25 to 35 per cent, and a high humidity of 90 per cent or above. Analysis for carbon

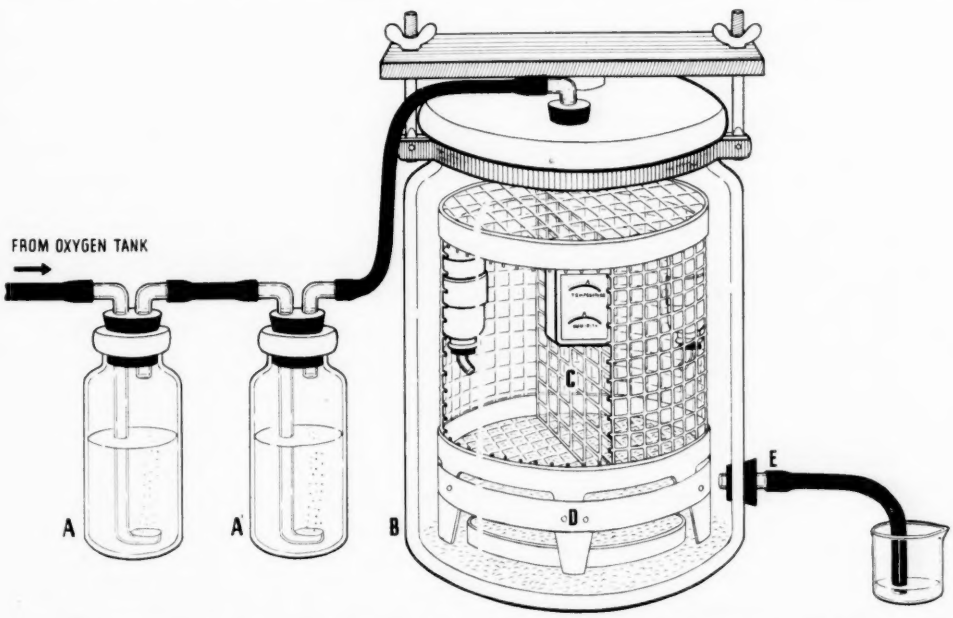

Fig. 1.—Diagram of apparatus used. *A* and *A'*, 500 ml. bottles for humidifying or dehydrating air or oxygen; *B*, 5-gallon glass jar with air-tight cover; *C*, divided wire cage with thermometer and humidity recorder attached; *D*, wire stand for $CaCl_2$; *E*, outlet tube.

dioxide by the Haldane apparatus showed the concentration to be kept below 2½ per cent. This was done by varying the stream of oxygen or air. This concentration of carbon dioxide is well below that which might produce toxic effects.[16] Analysis for oxygen showed it to be above 96 per cent in all oxygen experiments.

Each experiment ran for ten consecutive days or until death of all the rats. On each day the rats were removed from their cages for twenty minutes to allow for weighing of the animals and cleaning of the cages. At the end of the ten days the rats were removed from the chambers and kept in the laboratory for at least two more days to see if any died. All that died were autopsied and checked for pathologic changes of oxygen poisoning.

RESULTS AND DISCUSSION

For the experiments on air, ten to twelve rats were used for each temperature and humidity. None of the rats died. The gain in weight curve

(Fig. 2) was nearly normal with the exception of that for the rats kept at 90° F. and below 20 per cent humidity. There seems, then, to be nothing in the procedure which would injure the health of the rats with the possible exception of the combined high temperature and low humidity in one experiment.

In Table I and Fig. 3 the results of high oxygen concentration at two temperatures and three humidities are recorded. It can be seen from these data that oxygen had an unfavorable effect on both weight and life of all the groups of animals studied. Our observations confirm those of previous workers, that a high temperature is unfavorable.[11, 12] This can be seen from the number surviving at each of the humidities studied. At humidity 90 per cent there is a chance of 1 in 100,000 that the difference between 80 and 90° F. is not significant. At humidity 30 per cent the chance is less than 1 in 1,000,000 that

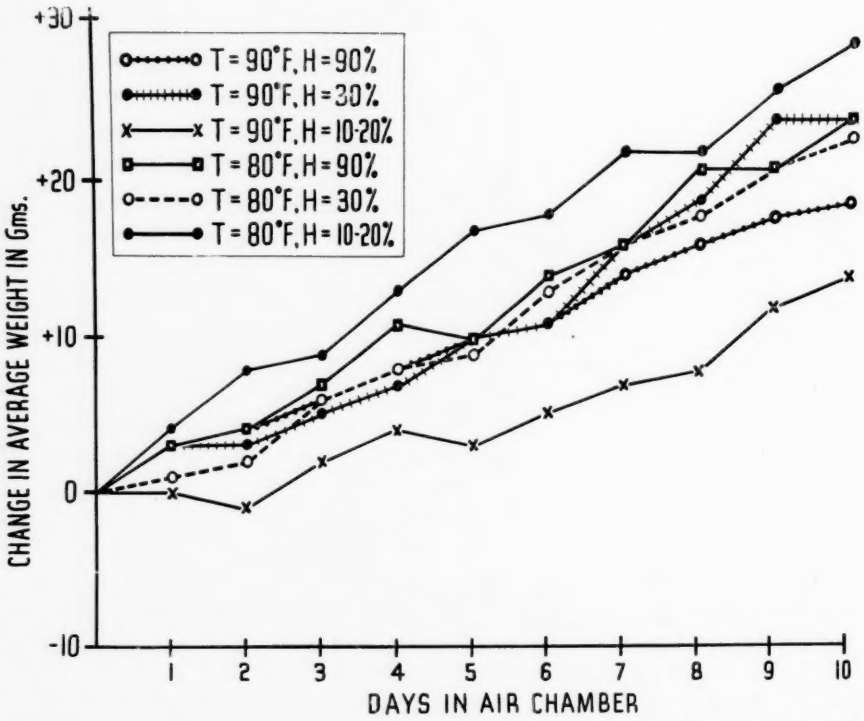

Fig. 2.—Effect of temperature and humidity on weight. The rats were kept in the chamber illustrated in Fig. 1 but in air instead of oxygen.

the difference is not significant. At humidity 20 per cent the chance that the difference is not significant is less than 1 in 100. If the best survival rate at temperature 90° F. is compared with the worst at temperature 80° F., it can be seen that the chance is very slight that there is any difference between these two groups. Temperature 80° F. and humidity 30 per cent gave the best results in the six groups studied. The chance that this does not differ from temperature 80° F. and humidity 90 per cent is less than 1 in 100. The chance that humidity 30 per cent does not differ from humidity less than 20 per cent is about 1 in 10,000. At ten days there is no evidence that the difference between 20 per cent humidity and 90 per cent at 80° F. is significant. However,

TABLE I

THE EFFECT OF OXYGEN ON THE SURVIVAL AND WEIGHT OF RATS

DAYS IN OXYGEN	T = 80° F. H = 90%		T = 80° F. H = 30%		T = 80° F. H = <20%		T = 90° F. H = 90%		T = 90° F. H = 30%		T = 90° F. H = <20%	
	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.
0	23	85	28	85	14	82	16	89	24	88	16	85
1	23	83	28	87	14	86	14	89	24	87	14	79
2	22	78	28	84	13	79	7	75	21	81	6	73
3	21	74	28	78	9	74	2	75	10	78	0	78
4	19	70	28	75	5	70	2	67	9	78	--	--
5	17	71	28	76	5	72	2	66	9	80	--	--
6	17	71	27	81	5	71	1	66	6	80	--	--
7	16	74	27	80	5	71	1	78	6	82	--	--
8	16	74	27	80	5	71	1	80	6	81	--	--
9	16	73	27	79	5	71	1		6	78	--	--
10	16	73	27	79	5	70	1		6	80	--	--
2 days after removal	14		26		5		1		6			

The average weight applies not only to those living, but also to the rats that died in the preceding twenty-four hours.

T, Temperature; H, Humidity.

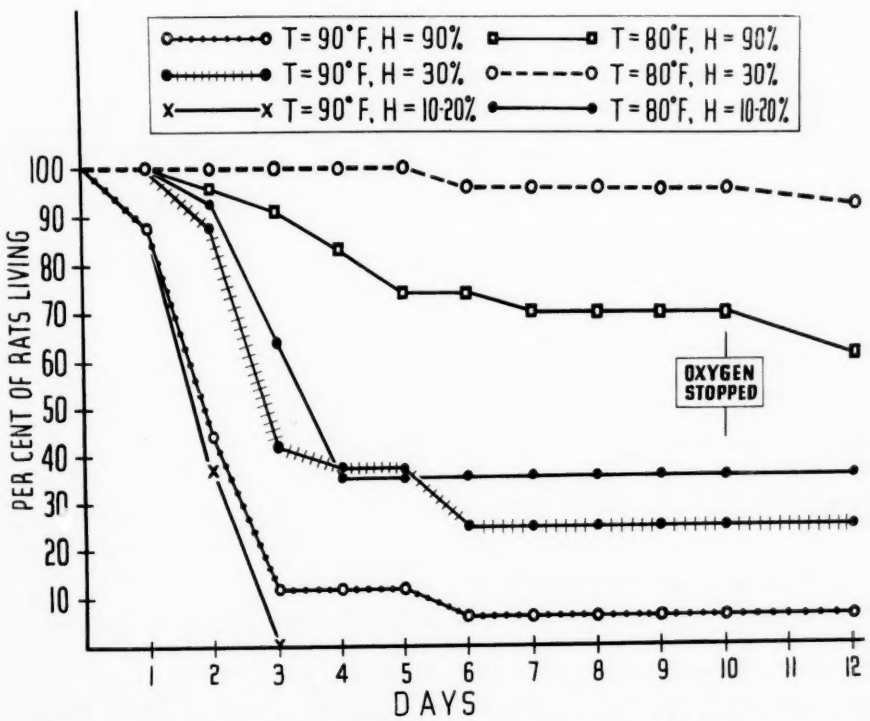

Fig. 3.—Survival of rats in an atmosphere of 96+ per cent oxygen for ten days.

if a comparison is made of the number dead on the fourth day, the chances are about 1 in 300 that the difference is not significant. This would seem to indicate that there is a difference in death rate although not a significant difference in total deaths over a period of ten days. For temperature 90° F. the best results were also obtained at 30 per cent humidity. Chances are about

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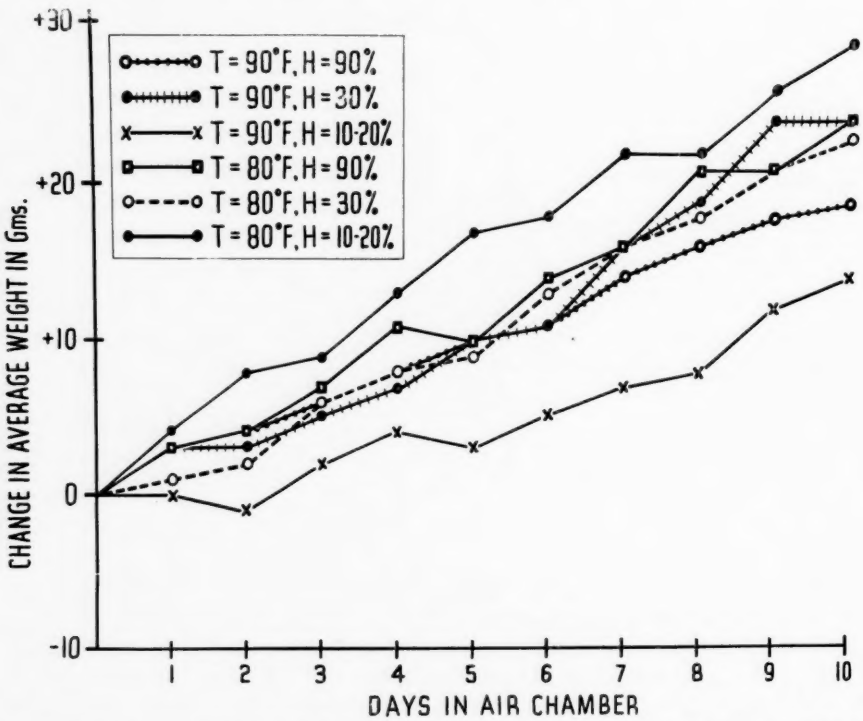

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	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.
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2	22	78	28	84	13	79	7	75	21	81	6	73
3	21	74	28	78	9	74	2	75	10	78	0	78
4	19	70	28	75	5	70	2	67	9	78	--	--
5	17	71	28	76	5	72	2	66	9	80	--	--
6	17	71	27	81	5	71	1	66	6	80	--	--
7	16	74	27	80	5	71	1	78	6	82	--	--
8	16	74	27	80	5	71	1	80	6	81	--	--
9	16	73	27	79	5	71	1		6	78	--	--
10	16	73	27	79	5	70	1		6	80	--	--
2 days after removal	14		26		5		1		6			

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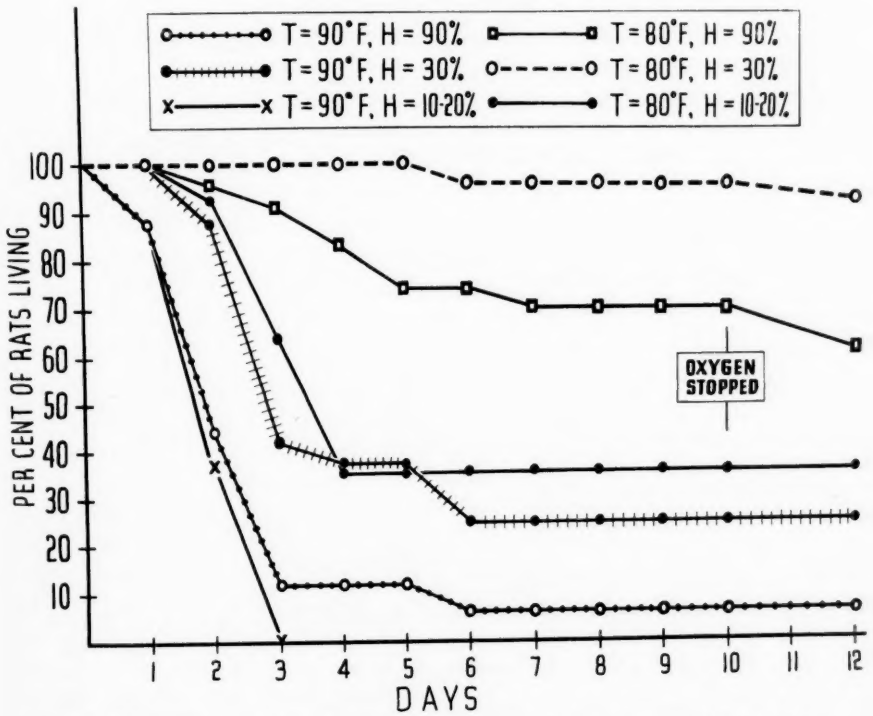

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1 in 200 that the humidity below 20 per cent is not worse than at 30 per cent. The chances that humidity 90 per cent is not worse than humidity 30 per cent are about 1 in 15.

CONCLUSIONS

A temperature of 90° F. is unfavorable for rats in high concentrations of oxygen when compared with a temperature of 80° F. at the three relative humidities studied. Humidity of 30 per cent is more favorable than humidities below 20 per cent or above 90 per cent. In the control of oxygen toxicity both the temperature and humidity are important.

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THE VARIABILITY OF HEART RATE AND BLOOD PRESSURE IN SELECTED GROUPS OF COLLEGE AND HIGH SCHOOL STUDENTS

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IF CARDIOVASCULAR measures are to be used to identify persons in good and poor physical condition or to measure progress during the course of a training program, these measures must have sufficient reliability and discriminatory power. Individuals are known to differ from each other as to resting and postexercise heart rate and blood pressure. It is also known that each individual varies from day to day and during the course of the day. In order to be able to distinguish one individual or group from another, it is necessary to have a comparatively wide range of variability among individuals or groups. Furthermore, repeated measures on the same individuals should show relatively small fluctuations. Otherwise the obtained differences between individuals could very well be due to chance variations in the individuals concerned or to errors in measurement. The measurable effects of a training program or medically prescribed treatments are likely to be quite small. For this reason any objective measure of progress should be highly reliable, that is, not subject to large chance variations. It is the purpose of this investigation to determine which of several commonly used cardiovascular measures are most reliable and which of them have the greatest discriminatory power.

Procedure.—Two groups of data were collected. The first was obtained from twenty college men and forty college women. One-half of the men and one-half of the women participated regularly in vigorous physical activity. The others reported little or no vigorous exercise. The second group of data was obtained from five 15-year-old high school boys who had an initial resting pulse between 70 and 80 beats per minute.[1]

Each of the college subjects came to the laboratory on four different days, usually at weekly intervals and at the same time of day. After five minutes of bed rest the pulse was counted for fifteen seconds, and the blood pressure (systolic and diastolic) was determined according to standard clinical procedures. The resting pulse and blood pressure determinations were repeated until consecutive readings were approximately the same. In only a few cases was it necessary to take a third set of readings, indicating that five minutes of bed rest were usually sufficient to stabilize the pulse and blood pressure at a resting level. The subjects then rode a stationary bicycle for one minute at moderate speed (60 revolutions per minute). A one-half-minute interval was allowed for resuming the reclining position and adjusting the cuff of the sphygmomanometer. Reclining pulse and blood pressure determinations were made one-half minute after the exercise was completed and again after two and four minutes had elapsed. This whole procedure was repeated on different occasions until four sets of readings were recorded for each subject.

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Received for publication, April 10, 1944.

In the case of the high school boys, heart rates were recorded each school day morning during a period of five months. The sitting pulse was counted before and after a standard stool stepping exercise (40 steps per minute for one minute, using a thirteen-inch stool). The postexercise pulse was counted continuously for two minutes, starting immediately after the cessation of the exercise.

Individual Means and Variability.—The original scores for the resting heart rate, the individual means arranged according to resting heart rate, and the averages for the vigorously and moderately active groups of college men and women are shown in Fig. 1. The corresponding data for the pulse one-half minute after exercise and for the increase due to exercise appear in the same order in Figs. 2 and 3. It is evident by inspection that the range of scores for

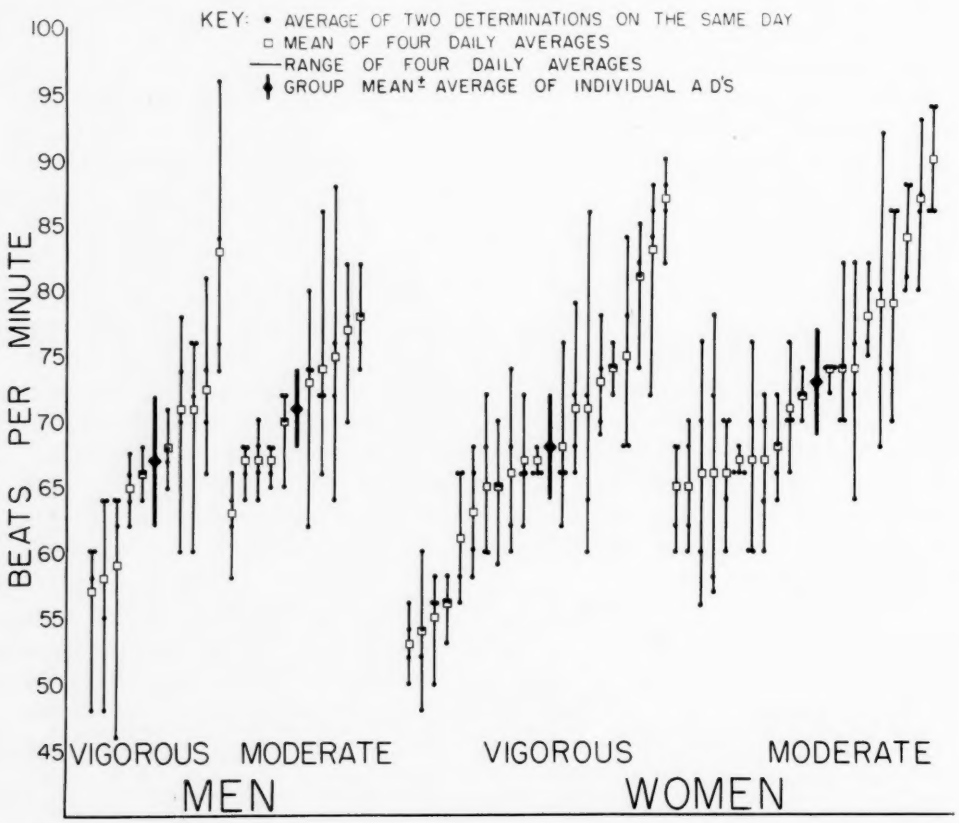

Fig. 1.—Resting heart rate for vigorously and moderately active groups of college men and women.

individuals covers a large part of the range for the group; also, that the rank order established in Fig. 1 does not hold for the postexercise pulse or for the increase due to exercise, that is, the correlation with resting heart rate is not very high.

High, low, and average values for the average deviation of single measures from the means for individuals are shown in Table I. The men and women are about equally variable, averaging from 4 to 5 beats per minute in heart rate and

from 3 to 5 mm. of mercury in blood pressure. The day-to-day average deviations in the heart rate of the 15-year-old boys ranged from 2 to 7 beats per minute and yielded averages which were about the same as those for the college groups.

The average deviation from the mean includes 57 per cent of the scores in a normal distribution, whereas 43 per cent of the scores fall beyond the limits set by the average deviation. Thus if a person's average resting heart rate was

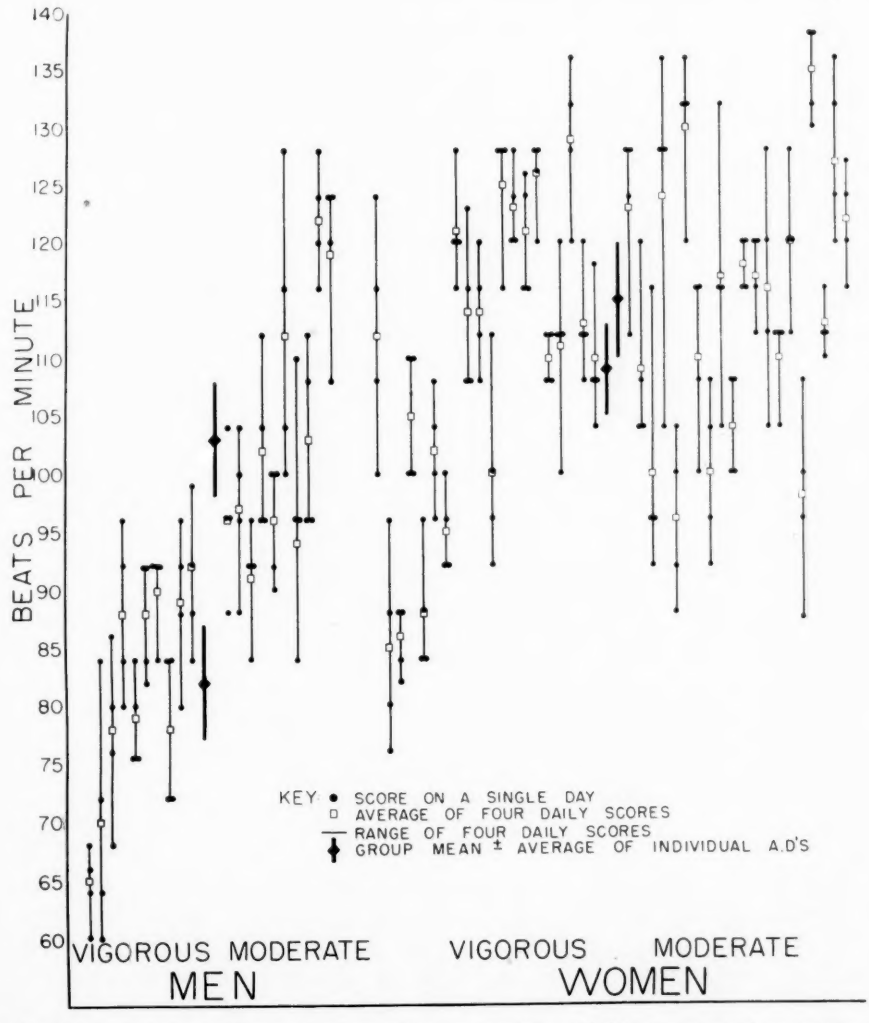

Fig. 2.—Heart rate one-half minute after moderate exercise. (Subjects arranged as in Fig. 1.)

75 beats per minute and his average deviation was 6 beats, the chances are that about three-fifths of any subsequent scores would be between 69 and 81 beats per minute. About one-fifth of the scores would be greater than 81 and another fifth would be less than 69. It would seem that fluctuations of this magnitude are altogether too large to warrant the use of single scores as measures of physical condition.

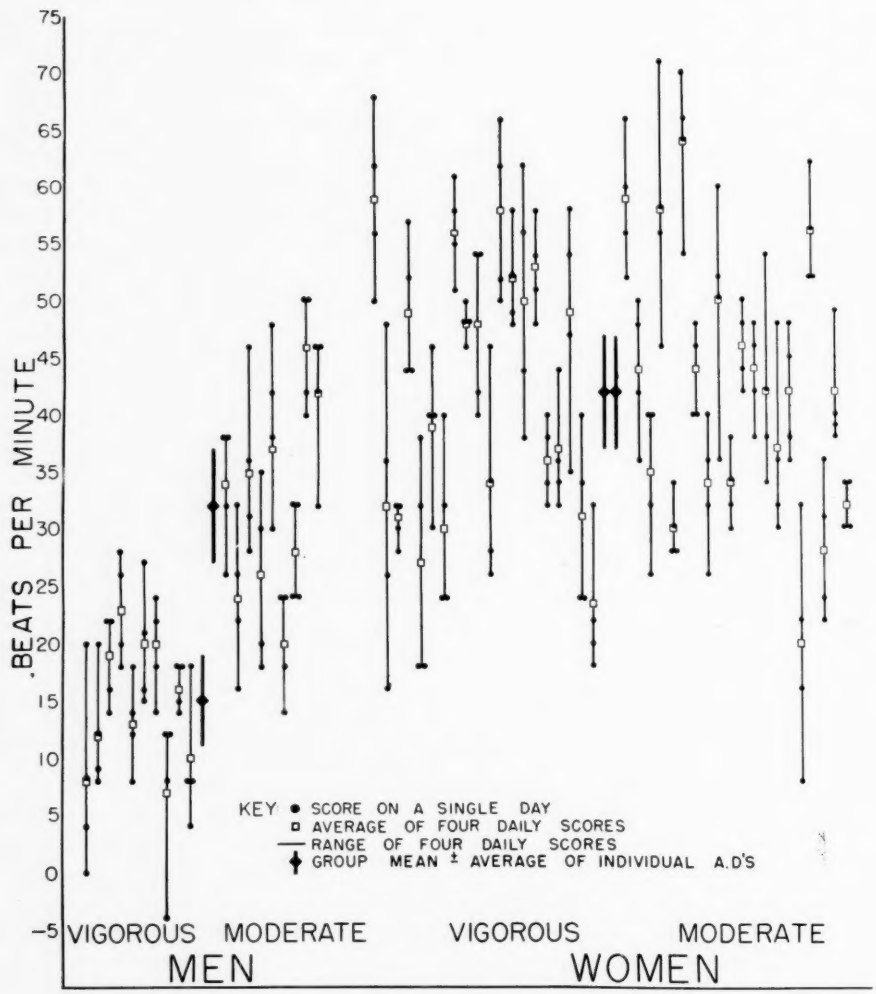

Fig. 3.—Heart rate increase due to moderate exercise. (Subjects arranged as in Fig. 1.)

TABLE I

HIGH, LOW, AND MEAN VALUES FOR THE AVERAGE DEVIATION FROM INDIVIDUAL MEANS

	RESTING			ONE-HALF MINUTE AFTER EXERCISE			INCREASE DUE TO EXERCISE		
	HIGH	LOW	MEAN	HIGH	LOW	MEAN	HIGH	LOW	MEAN
Heart Rate									
Men	8	1	4	8	3	5	7	2	5
Women	9	1	4	10	2	5	11	1	5
Systolic Pressure									
Men	5	1	3	9	1	5	10	1	5
Women	5	1	3	12	1	5	11	1	5
Diastolic Pressure									
Men	6	0	3	9	1	4	10	1	4
Women	7	1	3	7	1	3	9	0	4

Greater stability may be obtained by using the average of several scores instead of a single score. The variability of an average is equal to the average deviation divided by the square root of the number of scores ($A.D._M = \frac{A.D.}{\sqrt{N}}$). Thus the average deviation of four determinations is only half as variable as single scores. If the average deviation of the average of four scores is 3 beats per minute, the chances are that three-fifths of subsequent averages would be within 3 beats of the first average. It is also possible to reduce variability by more rigidly controlling the conditions under which the data are obtained.

Variation of Individuals from Group Means.—If a measure is to discriminate among individuals, the average deviation of individual means from the group mean must be large relative to the variability of the individual from his own mean. Such relationship can be indicated by means of reliability coefficients, but they are also evident from the figures shown in Table II. The average deviation in heart rate from the group mean is greatest one-half minute after exercise. The resting pulse has the least discriminatory value; that is, individuals are more nearly alike with respect to resting pulse than with respect to post-exercise pulse or pulse increase due to exercise. The variability in blood pressure is somewhat greater around group means than it is around individual means, ranging from 3 to 7 mm. of mercury in the case of the men, from 4 to 10 in the case of the women. Group variability in blood pressure is greatest one-half minute after exercise for both men and women.

TABLE II

HEART RATE AND BLOOD PRESSURE SCORES FOR COLLEGE MEN AND WOMEN AND AVERAGE DEVIATIONS FROM INDIVIDUAL AND GROUP MEANS

	RESTING			ONE-HALF MINUTE AFTER EXERCISE			INCREASE DUE TO EXERCISE		
	GROUP MEAN	A.D. FROM		GROUP MEAN	A.D. FROM		GROUP MEAN	A.D. FROM	
		$IND._M$	$GROUP_M$		$IND._M$	$GROUP_M$		$IND._M$	$GROUP_M$
Heart Rate									
Men	69	4	5	93	5	11	24	5	9
Women	70	4	7	112	5	10	42	5	9
Systolic Pressure									
Men	120	3	5	145	5	7	25	5	6
Women	107	3	6	140	5	10	33	5	8
Diastolic Pressure									
Men	75	3	6	72	4	7	−3	4	3
Women	68	3	5	65	3	6	−3	4	4

Even though there is considerable variation in heart rate from week to week, it can be shown by means of the F-test that all the pulse scores obtained from the subjects in our groups of college men and women could not have been obtained from the same individual.[2]

In order to determine the number of times that members of our experimental groups could be distinguished from each other on the basis of four pulse scores, the t-test technique was used to determine how large the differences between the means for any two individuals must be in order to be sure at the 1 per cent and the 5 per cent levels of confidence that the difference was significant.[3] In the case of the resting pulse for men, a difference of 17 beats is needed in order to

be certain at the 1 per cent level of confidence that the difference is not due to chance. Differences of 11 beats per minute are significant at the 5 per cent level of confidence.

Differences as large as 17 beats per minute in the resting pulse occurred only 15 times out of a possible 190 in our group of twenty men. Thus the differences in resting pulse would be significant at the 1 per cent level of confidence only 8 per cent of the time. Differences of 11 beats occurred 52 times (27 per cent of the time). Ten of these differences (5 per cent of 190) would have been significant on the basis of chance alone. The remaining forty-two cases represent the efficiency of the resting pulse in discriminating at the 5 per cent level among individuals in our group of twenty college men.

The pulse differences required for men and women at the 1 per cent and the 5 per cent level, the number of times differences as large as these occurred, and the percentage of differences meeting the 1 per cent and the 5 per cent requirement are shown in Table III. The greatest discriminatory power is shown by the pulse one-half minute after exercise for men. Of the 190 differences between individuals in this group, 77 (41 per cent) were large enough to be significant at the 1 per cent level of confidence and 117 (62 per cent) were large enough to be significant at the 5 per cent level of confidence.

TABLE III

THE OCCURRENCE OF HEART RATE DIFFERENCES LARGE ENOUGH TO BE SIGNIFICANT AT THE 1 PER CENT AND THE 5 PER CENT LEVELS OF CONFIDENCE

	TWENTY MEN (190 PAIRINGS)			FORTY WOMEN (780 PAIRINGS)		
	RESTING PULSE	ONE-HALF MINUTE AFTER EXERCISE	INCREASE DUE TO EXERCISE	RESTING PULSE	ONE-HALF MINUTE AFTER EXERCISE	INCREASE DUE TO EXERCISE
Population variance based on individual variability	41.1	45.2	34.4	32.6	47.7	49.2
Standard error M_1-M_2	4.53	4.75	4.15	4.05	4.88	4.96
Difference required at 1 per cent level	17	18	15	15	18	18
Difference required at 5 per cent level	11	11	10	10	12	12
Number of differences meeting 1 per cent requirement	15	77	73	197	251	223
Number of differences meeting 5 per cent requirement	52	117	112	356	413	391
Per cent of differences meeting 1 per cent requirement	8	41	38	25	32	29
Per cent of differences meeting 5 per cent requirement	27	62	59	46	53	50

Since some individuals are more variable than the average with respect to pulse scores, some of the differences as large as those specified in Table III will not yield a significant t-ratio (it equals the difference between two means divided by the standard error of that difference). Conversely when the standard error of the difference is small, differences less than the stated number of beats will be significant. These discrepancies balance each other however, so that the actual number of significant differences is approximately the same as the number of differences predicted in Table III. This was checked em-

pirically after computing a series of t-tests for the significance of the differences in pulse one-half minute after exercise among the twenty college men.

This analysis shows that the resting pulse has some discriminatory value, yielding more significant differences than would be expected by chance. It also shows the extent to which the pulse after exercise is superior to the resting pulse. And finally it calls attention to the limitations of even the best of the cardiovascular tests used in this experiment; the average of four pulse scores one-half minute after a standard exercise was able to distinguish only 41 per cent of the time at the 1 per cent level of confidence and 62 per cent of the time at the 5 per cent level.

Reliability Coefficients.—The discriminatory power of a test can also be inferred from the coefficients obtained when two sets of scores on the same subjects are correlated. Such coefficients become larger with an increase in the range of scores around the group mean, and as the variability with respect to individual means decreases.

The reliability coefficients obtained from our data on the twenty college men and forty college women are given in Table IV. The coefficients under the heading I II were obtained by correlating the first set of scores with the second. The reliability of the average of four determinations (r_{44}) was estimated from $r_{(I\,II)\,(III\,IV)}$ by means of the Spearman-Brown formula.[4]

If the degree of reliability represented by a coefficient of .85 or above is desired in cardiovascular testing, it is necessary to use the average of four determinations rather than single test scores.

TABLE IV

RELIABILITY COEFFICIENTS FOR SINGLE DETERMINATIONS OF HEART RATE AND BLOOD PRESSURE AND FOR THE AVERAGE OF FOUR DETERMINATIONS

	RESTING		ONE-HALF MINUTE AFTER EXERCISE	
	$r_{I\,II}$	r_{44}	$r_{I\,II}$	r_{44}
Heart Rate				
Men	.63	.83	.86	.94
Women	.76	.86	.78	.90
Systolic Pressure				
Men	.74	.87	.60	.78
Women	.88	.93	.74	.88
Diastolic Pressure				
Men	.71	.86	.71	.87
Women	.72	.86	.76	.92

CONCLUSIONS

1. Pulse after a standard exercise is a more reliable measure than the resting pulse, but the resting systolic blood pressure is a more reliable measure than the systolic pressure after exercise.

2. Even when conditions are carefully controlled, an individual's heart rate and blood pressure are so variable from day to day that a number of determinations must be made if his general status is to be established.

3. Cardiovascular tests in general have little discriminatory power because the differences in scores among individuals are small in relation to individual variability.

4. Individuals can more clearly be distinguished from each other on the basis of postexercise pulse rates than on the basis of resting heart rate or the increase due to moderate exercise. The same is true of postexercise systolic and diastolic blood pressures.

5. The relative efficiency of heart rate scores in distinguishing individuals from each other has been demonstrated in terms of the percentage of significant differences among individuals in a group. Whereas only 8 per cent of the differences in the resting pulse for the twenty men in our experiment are significant at the 1 per cent level of confidence, 41 per cent of the differences in pulse one-half minute after exercise are significant at this level. The corresponding figures for the women are 25 per cent and 32 per cent. At the 5 per cent level of confidence, 62 per cent of the differences in pulse after exercise are significant among the men; among the women only 53 per cent of the differences are significant at this level of confidence.

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THE OSCILLOMETER AND THERMOCOUPLE AS DIAGNOSTIC AIDS IN PERIPHERAL VASCULAR DISEASE

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THE widespread use of the oscillometer and thermocouple as diagnostic aids in peripheral vascular disease occasioned this study. An attempt was made to record the evidence obtained by various diagnostic procedures and to correlate this evidence with the circulation in the extremities examined.

TECHNIQUE

To maintain standard conditions for all the observations, an air-conditioned room was equipped in the Falk Clinic. In this room it was possible to maintain both the temperature and the humidity within a narrow range. A temperature of 75° F. and a relative humidity of 75 per cent were used as standard, and all observations were made under these conditions.

Data relative to the peripheral circulation were obtained on 102 subjects. Of these, 48 were normal individuals, 8 were asymptomatic with relation to peripheral vascular disease although they had some other systemic disease, 6 had thromboangiitis obliterans, and 28 had arteriosclerotic vascular disease. Of the 12 remaining patients, varicosities, neuritis, and angina pectoris accounted for all but 1. This patient was a Negress in whom no arterial pulse could be obtained in the upper extremities.

The sex, age, and race and the location, character, and duration of the symptoms of each patient were noted (Fig. 1). Previous treatment received by the patient and measures that afforded relief from symptoms as well as the family and past medical history were recorded. Physical examination, including a neurological examination, was given each patient.

Alterations in the color of the lower extremity in the dependent, horizontal, and elevated positions were noted. A period of ten minutes in each of these positions was allowed before the color change was recorded. Abnormal pigmentation, the presence or absence of phlebitis, ulcers, gangrene, edema, trophic changes in the skin, nails, and hair, and pulsations of the dorsalis pedis and posterior tibial arteries were observed. Calf measurements were recorded at the level of the greatest circumference.

Skin temperature observations were obtained with a skin temperature thermocouple apparatus in the following manner. With the subject in the sitting position, the lower extremities were exposed in the air-conditioned room

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The Westinghouse Electric Supply Company equipped the air-conditioned room at the Falk Clinic on the recommendation of Dr. T. Lyle Hazlett, Medical Director of Westinghouse Electric & Manufacturing Co.

The subjects used in this study were largely taken from the diabetic and medical services of the Falk Clinic and from the student body of the University of Pittsburgh. Additional patients were seen through the courtesy of Dr. T. Lyle Hazlett, Dr. Joseph H. Barach, Dr. W. Leigh Cook, Jr., Dr. W. M. McClements, and Dr. L. L. Pennock.

Received for publication, May 12, 1944.

for a period of twenty-five minutes. Skin temperatures were then recorded from the anterior portion of the thigh, the patella, the mid-calf, the internal and external malleoli, the dorsum of the foot, and the mid-plantar surface and from the skin at the base of the great toenail. The legs were then placed in the horizontal position upon a low, padded stool. After they had remained in this position for ten minutes, skin temperatures were again recorded from the areas noted above. Finally the patient was placed in the supine position upon an examining table with the lower extremities supported at an angle of 45 degrees above the horizontal. Skin temperatures were again recorded after a similar stabilizing period. The skin temperature findings from the thigh to the great toe for each subject in each of the three positions were plotted to illustrate the centrifugal variation in skin temperature.

A recording oscillometer was used to obtain the oscillometric readings. The cuff was placed about the calf and inflated to a level somewhat above the systolic blood pressure. The pressure in the system was then decreased by 10 to 20 mm. Hg in successive steps until no pulsation was noted.

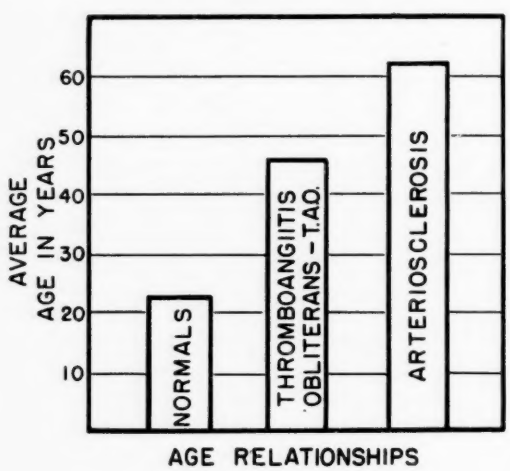

Fig. 1.

Vibratory sensation was tested according to the method of Barach.[1] An especially constructed tuning fork was used which made it possible to observe the instant when a standard amplitude of vibration was obtained. The length of time which elapsed from the instant the standard amplitude of vibration was reached until the subjective sensation disappeared was recorded with a stop watch. Vibratory sensation determinations were made at the angle of the sternum, the lower end of the radius and ulna, the anterior superior spine of the ilium, the mid-point of the tibia, the internal and external malleoli, and over the tip of the sacrum. These readings were then individually plotted against the normal variation previously obtained by Barach.

At the suggestion of Dr. C. C. Guthrie, the calorimetric method of estimating the blood flow in the extremities devised by Stewart[14] was used. Through the courtesy of Dr. J. M. Rogoff, the original calorimeters which Stewart devised were available, and with these as models, suitable calorimeters were constructed. The technique described by Stewart was rigidly followed.

This technique is summarized[11] briefly. The subject to be examined placed the extremities in a bath containing water at approximately 30° C. At the end of fifteen minutes the part to be tested was transferred swiftly and with a minimum of exposure into a calorimeter containing water at exactly the same temperature as that in the preliminary bath. The water within the calorimeter was stirred by means of feathers, and temperature readings estimated to the hundredth of a degree were taken at regular intervals for twenty minutes.

From the transfer of heat from the part being tested to the water bath it was possible to estimate the flow of blood through the part by the following formula:

$$Q = \frac{H}{T - T^1} \times \frac{1}{S}$$

Where Q is equivalent to the quantity of blood flowing through the part during the period of observation; H is the heat given off to the calorimeter during the period of observation and the water equivalent of the calorimeter and of the part. T represents the temperature of the arterial blood. Stewart's figure for this is 0.5° C. below the rectal temperature or the equivalent of oral temperature. T^1 equals the temperature of the venous blood. Because of the heat transfer that takes place between the water in the calorimeter and the superficial veins, the temperature of the venous blood was taken to be equal to the mean temperature of the calorimeter. S represents the specific heat of blood, or 0.9.

The volume of the part through which the blood flow was being determined was measured by the displacement of water. The flow of blood was recorded in grams of blood per 100 c.c. part volume per minute. As pointed out by Sheard,[12] the actual determination of the volume flow of blood by this method is open to criticism.

OBSERVATIONS

Of the 48 normal subjects in the group examined, 39 were men and 9 women. The average age of the normal group was 23 years. All 6 with thromboangiitis obliterans were males; they had an average age of 45.5 years; 3 members of this group were Hebrew. The average age of the 28 individuals with arteriosclerosis was 61.7 years; of this group 14 were men and 14 women (Fig. 1).

In the arteriosclerotic group 23 of the 28 patients were diabetic. A history of neuritis was obtained in one-half of the patients who were diabetic. Either history or evidence of an active phlebitis was present in 3 of the 6 patients with thromboangiitis obliterans. Only 1 of the 28 subjects with arteriosclerosis gave a history of phlebitis. None in the normal or asymptomatic group had had phlebitis.

In the subjects with arteriosclerosis of the lower extremities, intermittent claudication was a common symptom; almost as frequently the pain was noted in the ankle, dorsum, longitudinal arch, or in the toes. Numbness, tingling, burning, aching, and rest pain were common symptoms in those in this group. A sense of local fatigue rather than intermittent claudication was often an early sign of peripheral vascular involvement. Of the patients with arterio-

sclerotic vascular disease who exhibited rest pain, almost 50 per cent were able to obtain relief from the pain by moderate exercise, warmth, or massage. None of the patients with thromboangiitis obliterans found the rest pain relieved by exercise. Both the patients in the arteriosclerotic group and those with Buerger's disease obtained relief from intermittent claudication by rest.

A variable decrease from the normal calf measurement was noted in subjects with peripheral vascular disease (Fig. 2). Atrophic changes in the skin were noted in over 50 per cent of those with arteriosclerosis. Only 25 per cent of this group had a normal distribution of hair over the extremities. As would be expected from the variation in the age groups, the subjects with arteriosclerosis had a higher average blood pressure than did those with Buerger's disease.

Palpable varicosities were noted in over 75 per cent of the subjects with symptoms of arteriosclerotic vascular disease. One-half of this group consisted of older women, many of whom had had large families; this probably accounts for the high incidence of variocosities. However, it is not unlikely that the symptoms of vascular disease are more prone to appear in the presence of both varicosities and arteriosclerosis.

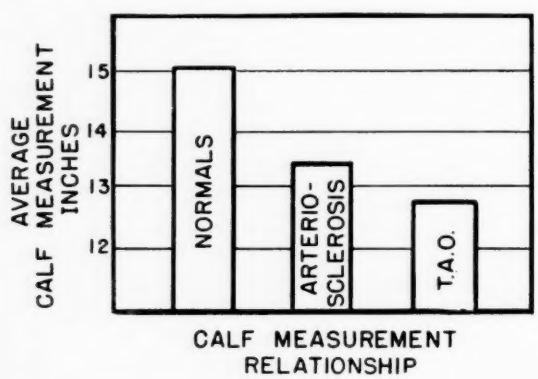

Fig. 2.

Merely the presence of rubor, cyanosis, or pallor is not of itself indicative of vascular disease. Included in this series were both normal and symptomatic subjects with these signs. Rubor was commonly seen in normal individuals with the feet in a dependent position when the room temperature exceeded 78° F. Edema of the lower extremities of some degree was found in 45 per cent of those in the arteriosclerotic group. However, in interpreting this figure it must be recalled that one-half of those in this group also had palpable varicosities. Three of the patients with thromboangiitis had demonstrable edema of the legs.

The pulsation of the dorsalis pedis artery was palpable in all but 2 of the normal individuals; significantly it was also palpable in over one-half of those with arteriosclerotic vascular disease. This pulsation was present most frequently in those with mild evidence of vascular disease. The posterior tibial pulsation was absent frequently in the normal subjects, but was palpable in over 20 per cent of those with arteriosclerosis. It was noted that when the posterior tibial pulsation was not palpable, it could frequently be visualized by everting the foot. This evidence supports the well-known clinical conclusion

that the absence of the dorsalis pedis or posterior tibial pulsation does not necessarily indicate the presence of vascular disease; and, conversely, the presence of a palpable pulsation does not rule out the possibility of arterial vascular disease.

The oscillometric findings did not indicate unequivocably the presence or degree of involvement of peripheral vascular disease. The average reading obtained in patients with symptoms of arteriosclerotic vascular disease were slightly higher than those obtained in normal individuals (Fig. 3). This may be explained by the fact that in those with chronic arterial disease the musculature of the leg is weak and somewhat atrophic. The artery may then be somewhat superficially situated and each impulse of the artery is transmitted more readily to the inflated cuff than it is in the individual with rigid, heavy musculature. In the presence of arteriosclerosis, if the hardened artery is superficially located, it may rise out of its bed with each thrust of the pulse; this motion may be transmitted to the oscillometer and recorded as a normal arterial pulsation when actually the blood flow to the part is inadequate due to the diminished lumen of the vessel. Conversely, diminished oscillometric

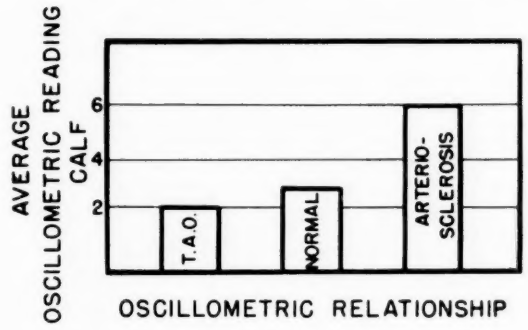

Fig. 3.

readings may be obtained in normal individuals with heavy musculature because the arterial impulse is poorly transmitted as the artery lies in a bed of loose areolar tissue with firm muscular support.

While diminished oscillometric readings could not be interpreted as being absolutely indicative of vascular disease, it was possible with repeated oscillometric studies in the same individual to determine from time to time the changes in peripheral pulsation that occurred. The evidence obtained by repeated oscillometric observations on the course of a vascular disease, is, in our opinion, one of the chief uses of the oscillometer today.

The skin temperatures of all subjects in the dependent, horizontal, and elevated positions were recorded and no significant or constant changes were noted in any of these positions. No significant changes in skin temperature occurred in normal, arteriosclerotic, or thromboangiitic extremities with the legs in the dependent, horizontal, or elevated positions.

Of 384 observations on the leg in the dependent position, 424 in the horizontal, and 368 in the elevated position, it was found that the average skin temperature in each position was very slightly higher in the arteriosclerotic extremity than in the normal extremity.

Chronic vascular disease of the larger arteries does not necessarily result in decreased skin temperatures. The temperature of the skin is obviously dependent upon many factors, one of these being arteriolovenous anastomoses which shunt blood directly from the arterioles into the superficial veins.[6] By this means the blood may be directed into the cutaneous tissues before it has been cooled by passing through the capillary circulation. Since certain types of arteriosclerosis present pathologic lesions of the larger arteries but not, to any great extent, of the arterioles, it is entirely possible by means of this arteriolovenous shunt to have a normal skin temperature although the circulation of the part may be impaired. This mechanism is only one factor that renders the interpretation of skin temperatures difficult; the temperature of the examining room, the previous activity of the part, the clothing worn by the patient, the psychic state of the patient, and many other factors may cause reflex changes in the circulation of an extremity and thereby alter the skin temperature.

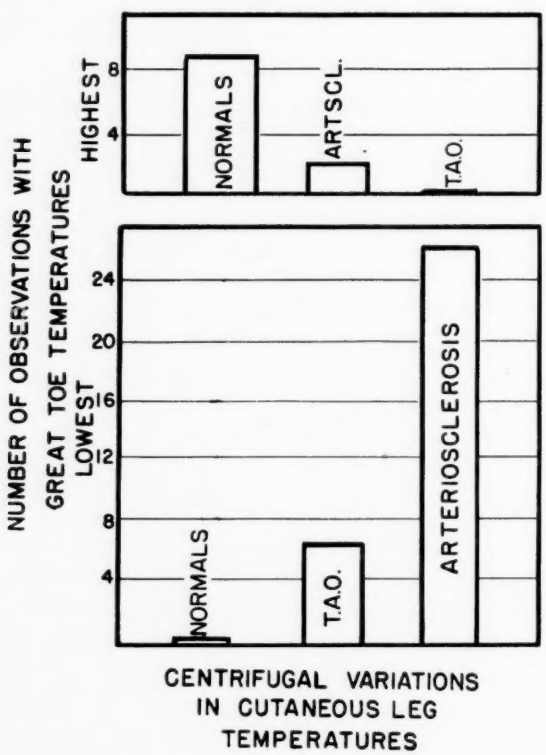

Fig. 4.

Possibly a more satisfactory apparatus than the thermocouple for determining skin temperature is the radiometer; this device has the advantage of measuring the radiated heat from a larger surface area. With the thermocouple device it is possible to unknowingly place the metallic contact close to a superficial vessel and thereby obtain an excessively high reading. It is our opinion that a reliable interpretation of a patient's skin temperature may be made by the examiner placing his hand briefly on the extremity in various locations.

Obviously the hand should be warmed to room temperature. The impression of the skin temperature that is obtained by palpation gives, in our opinion, a more accurate index of the circulatory status of the extremity than is obtained by the use of the thermocouple. From the data presented here it is apparent that normal skin temperatures as obtained with the thermocouple do not exclude arterial vascular disease.

It is generally recognized that there is a centrifugal decrease in skin temperature. It is interesting to note that in this study the temperature of the great toe was the highest recorded on the entire leg in 16 observations; in 40 individuals the great toe temperature was the lowest obtained; and in 46 the temperature of the great toe was somewhere between the highest and lowest recorded on that extremity (Fig. 4). The dorsum of the foot was the warmest part in 36 patients, 26 of these having arteriosclerotic vascular disease. Again the explanation of these alterations in skin temperature may lie in the altered cutaneous blood supply caused by changes in the arteriolovenous anastomoses. The centrifugal decrease in the temperature of the arterial *blood* is not disputed, but since the arteriolovenous anastomoses have been found mainly in the distal portion of the extremities, the increased cutaneous circulation may permit the *skin* to have a higher temperature peripherally than centrally.

Studies on the blood flow in the feet by Stewart's technique indicated that the blood flow in patients with arteriosclerotic vascular disease and thromboangiitis obliterans averages about two-thirds that obtained in normal subjects (Fig. 5). It is to be noted, however, that a wide range of values may be

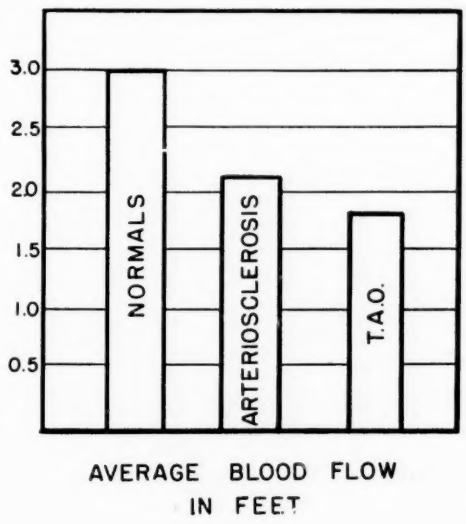

Fig. 5.

regarded as normal. Slight errors in technique or alterations in the environment markedly alter the results. To obtain comparable readings in a series of patients it is essential that they be examined under exactly the same conditions.

Sheard[12] has criticized this method of quantitatively estimating the circulation. For this reason no quantitative figures of blood flow are offered. Comparative blood flow readings that are very high or very low when obtained

under standard conditions may be interpreted readily, but readings in the wide borderline range of normal values cannot be used as an accurate index of the circulation of the part. Determination of the blood flow by this method is of little use clinically, and other probably more satisfactory methods are available for investigative purposes.

Estimation of vibratory sensation by the method described yields confirmatory evidence of deficient circulation. Of the individuals examined in this study, all those with obviously deficient circulation had diminished vibratory sensation. However, diminution in vibratory sense is a late sign of circulatory deficiency and is of little diagnostic value in early cases. It is probably a confirmatory sign of the nervous deterioration accompanying impaired circulation.

SUMMARY AND CONCLUSIONS

1. Observations relative to the peripheral circulation were made on 102 individuals. This group included 48 normal subjects, 8 asymptomatic, 6 with thromboangiitis obliterans, and 28 with arteriosclerosis. Observations as to history, symptoms, physical signs, oscillometric readings, skin temperatures, blood flow, and vibratory sensation were noted and the results tabulated.

2. The techniques used in obtaining the data are briefly described.

3. While in arteriosclerosis of the lower extremity, symptoms referable to the calf were most common, pain was noted almost as frequently in the ankle, arch, dorsum, or toes. Numbness, tingling, burning, aching, and rest pain were symptoms noted almost as frequently as intermittent claudication in arteriosclerotic vascular disease. A feeling of local fatigue was often the first sign of vascular disease.

4. Arteriosclerotic rest pain was often relieved by moderate exercise. The pain of thromboangiitis obliterans was not relieved by exercise in any of our patients.

5. Normal distribution of the hair over the lower extremity was noted in only 25 per cent of the patients with vascular disease.

6. Rubor, cyanosis, or pallor of an extremity may be present in the absence of arterial vascular disease.

7. The absence of the dorsalis pedis or posterior tibial pulsation was not pathognomonic of vascular disease, and the presence of a pulsation did not exclude vascular pathology.

8. The oscillometric readings were not necessarily found to be decreased in arteriosclerotic peripheral vascular disease; normal individuals with heavy musculature may have diminished oscillometric readings.

9. Normal skin temperatures may obtain in individuals with vascular disease.

10. Estimation of the blood flow by Stewart's calorimetric method indicated that the blood flow in patients with vascular disease averaged about two-thirds of that in normal subjects.

11. Estimation of the vibratory sense by the method of Barach yields confirmatory evidence of deficient circulation but is of little early diagnostic value.

12. The arteriolovenous anastomoses are suggested as one mechanism partially explaining the variations in skin temperature that occur in normal individuals and those with peripheral vascular disease.

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A STUDY ON THE RELATIONSHIP OF BLOOD ISO-AGGLUTININ TITERS TO TOTAL SERUM PROTEIN CONCENTRATION

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THE role of blood iso-agglutinins in whole blood and plasma transfusion reactions now is established. With the current renewal of interest in protein metabolism and the widespread use of plasma and protein hydrolysates in combating hypoproteinemia, the behavior of blood iso-agglutinin titers, with changes in serum protein concentration, assumes importance from a clinical and experimental standpoint. The present study demonstrates that no definite relationship exists between blood iso-agglutinin titers and total serum protein concentrations.

There are certain well-established facts regarding the behavior of blood iso-agglutinins in man. Minor variations in serum titers occur from time to time and without apparent cause in normal persons. The titer is reduced in middle-aged and elderly persons. Increased titers have been observed during acute infections, in heterospecific pregnancies,[5] and after plasma transfusions.[6] Davidsohn[4] reported very high titers in patients with sickness resulting from the administrations of horse serum and low titers in patients with severe anemia or chronic leucemia.

METHODS

Samples of blood for total serum protein determinations and titration of iso-agglutinins were collected according to the standards of Kagan,[1] which require that the blood be drawn within one minute after application of the tourniquet; the samples must be kept and centrifuged in well-stoppered tubes; only non-hemolyzed serum can be used, and no amount of fluid greater than 500 c.c. should be ingested at any time within four hours before taking the sample.

Total serum protein determinations were made with the Kagan proteinometer* by the falling drop method.[2, 3] A few determinations were carried out in the hospital clinical laboratory by the colorimetric method. The normal range of total serum protein concentration was considered to be from 6.0 to 7.5 Gm. per 100 c.c., as determined by Kagan.[1]

Titration of serum iso-agglutinins was performed by making successive dilutions of serum in 7 mm. test tubes with physiologic saline solution. To each test tube was added 3 drops of a 1.0 per cent suspension of A or B cells in physiologic saline. Fresh cell suspensions were prepared daily from two healthy adults. The cell-serum mixtures were shaken vigorously and incubated in a water bath at 37.5 C. for thirty minutes. Each tube then was centrifuged

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Supported by a grant from the Council on Pharmacy and Chemistry of the American Medical Association.

Received for publication, June 27, 1944.

*The Kagan proteinometer is manufactured by E. H. Sargent & Co., Chicago, Ill.

at 500 r.p.m. for one minute, after which the cells were resuspended by a gentle but thorough shaking. Titration end points were determined by low-power microscopic examination of the suspension. The highest dilution which contained clumps of three or more cells was regarded as the end point. Only differences of two or more dilutions were considered significant in individual patients who were observed at intervals.

Amigen* powder was administered by dissolving the preparation in hot broth. Dosage varied from 6 to 8 tablespoonfuls (from 54 to 72 Gm.) in approximately 200 c.c. of broth two to three times daily.

All technical procedures, except the few colorimetric protein determinations, were carried out by us.

CLINICAL MATERIAL

Normal persons were selected at random for these studies from the medical and nursing staffs and the medical classes of the University of California Hospital. All had serum protein concentrations within the expected normal range. With one exception, all the patients with hypoproteinemia were in the same hospital.

Three patients with hypoproteinemia received amigen orally to replenish depleted body protein stores and raise circulating serum protein levels. Two of these patients received whole blood transfusions repeatedly. In no instance were titrations performed earlier than three days after transfusion. Although no significant changes in titer have been observed subsequent to compatible blood transfusions, elevations have been noted after the injection of plasma. Wiener[7] suggests that the presence of red cell stroma may contribute to the immunization effect.

A. W., a 29-year-old white man, noted extreme weakness, pallor, and dyspnea six months prior to his entry to the hospital April 5, 1943. A diagnosis of aplastic anemia was established by examination of the peripheral blood and bone marrow. The initial blood count revealed the hemoglobin to be 1.5 Gm. (8 per cent) and the red blood cells to be 440,000 per cubic millimeter. The presence of severe hypoproteinemia was discovered by the finding of a total serum protein concentration of 4.27 Gm. per cent. The treatment during the initial hospital stay of one month consisted of whole blood transfusions, liver extract administered parenterally, ferrous sulfate, vitamin C, and amigen (from 54 to 72 Gm. twice daily) by mouth. During the initial period the patient developed purpura on one occasion and a number of large furuncles. The furuncles cleared rapidly. Upon discharge from the hospital the hemoglobin was noted to be 80 per cent. There were six subsequent hospital entries for transfusion of whole blood or red cell mass. The treatment that he received while ambulatory was similar to that which had been instituted in the hospital. Death occurred Oct. 11, 1943, from a cerebral hemorrhage during hospitalization for a recurrence of purpura. Autopsy confirmed the diagnosis of aplastic anemia.

H. W., a 46-year-old Jewish man, noted weakness for one month before entering the hospital March 18, 1943. Two weeks prior to admission he had been disturbed by dyspnea on exertion, tinnitus, and precordial pain. A blood count taken March 17, 1943, because of suspected lymphangitis of one arm revealed: hemoglobin, 4.6 Gm. (32 per cent); red blood cells, 1.64 million; and white blood cells, 2,250. Additional blood and bone marrow studies resulted in a diagnosis of aplastic anemia. The treatment for this patient was identical to that employed in the patient in preceding case, but purpura appeared early and

*Amigen is a dried enzymic digest of purified casein and pork pancreas containing amino acids and polypeptides kindly furnished by Mead Johnson & Co., Evansville, Ind.

rectal and gingival bleeding became severe. There was progressive reduction of the hemoglobin concentrations. The patient was discharged from the hospital June 30, 1943; the hemoglobin concentration was 94 per cent, and there was severe bleeding from the gums and extensive retinal hemorrhages. In spite of transfusions, bleeding increased and death occurred at home July 31, 1943. An autopsy was not performed.

H. S., a 48-year-old white man, was proved to be suffering from chronic glomerulonephritis in the nephrotic stage. Treatment consisted of a low salt, high protein diet which was supplemented with amigen in 54 to 72 Gm. doses twice daily. There was little change in the total serum protein concentration over a five-week period. The patient was discharged from the hospital May 20,1943, and failed to return for follow-up studies.

OBSERVATIONS

The twenty normal subjects reported in Table I had the usual distribution of blood types and a range of total protein concentrations within normal limits. In this group there was no constant relationship between iso-agglutinin titers

TABLE I

NORMAL SUBJECTS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (M. D.)	B	6.18	1:4096	
2 (M. A.)	O	6.28	1:1024	1:512
3 (A. B.)	A	6.30		1:1024
4 (D. G.)	O	6.39	1:4096	1:1024
5 (H. K.)	A	6.42		1:512
6 (S. M.)	O	6.46	1:8192	1:8192
7 (M. M.)	A	6.49		1:2048
8 (M. L.)	A	6.53		1:13384
9 (F. L.)	O	6.62	1:4096	1:2048
10 (B. G.)	O	6.62	1:4096	1:512
11 (J. R.)	O	6.62	1:8192	
12 (D. R.)	B	6.77	1:2048	
13 (M. S.)	O	6.90	1:2048	1:512
14 (N. C.)	O	6.97	1:65536	1:16384
15 (M. B.)	A	7.08		1:16384
16 (M. W.)	A	7.22		1:2048
17 (A. W.)	A	6.10		1:512
18 (M. S.)	O	7.03	1:4096	1:2048
19 (C. H.)	A	6.76		1:2048
20 (S. D.)	A	6.87		1:512

TABLE II

HYPOPROTEINEMIC SUBJECTS
SINGLE OBSERVATIONS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (A. C.)	B	4.74	1:512	
2 (A. B.)	A	4.97		1:1024
3 (L. S.)	B	5.56	1:256	
4 (O. U.)	O	5.59	1:256	1:512
5 (D. D.)	O	5.69	1:8192	1:1024
6 (P. C.)	A	5.71		1:16384
7 (B. C.)	A	5.72		1:2048
8 (F. S.)	A	5.82		1:1024
9 (B. L.)	O	5.82	1:2048	1:512

and serum protein levels. As has been noted by other investigators, anti-A titers were higher than anti-B titers. The high anti-A titer in one subject, 14 (N. C.), was checked repeatedly but remains unexplained.

Single determinations of iso-agglutinin titers in patients with hypoproteinemia are recorded in Table II. Hypoproteinemia was mild in the majority of patients and the titers of the entire group evinced no significant difference when compared with the normal subjects. No correlation existed between the degree of hypoproteinemia and iso-agglutinin concentrations.

TABLE III

PATIENT A. W.
TYPE O

DATE (1943)	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
3/24	5.37	1:1024	1:512
3/25	5.37	1:1024	1:512
4/7	4.26		
4/12	5.66	1:4096	1:2048
4/20	7.11	1:4096	1:1024
4/27	6.49	1:4096	1:1024
5/5	5.66	1:8192	1:2048
5/10	6.14	1:8192	1:4096
5/18	6.18	1:2048	1:4096
6/3	5.70	1:16384	1:4096
6/18	6.25	1:16384	1:16384
6/25	5.97	1:16384	1:16384
6/28	6.35	1:16384	1:8192
7/9	6.49	1:8192	1:8192
7/27	6.11	1:16384	1:16384
8/14	6.42	1:8192	1:8192
8/30	6.14	1:8192	1:8192
9/10	6.07	1:16384	1:16384
9/22	5.80	1:16384	1:16384
9/30	5.70	1:16384	1:16384
10/8	5.45	1:16384	1:16384

The iso-agglutinin titers in one patient (A. W. Table III) rose from the initial concentrations of anti-A 1:1,024 and anti-B 1:512 to concentrations of anti-A 1:8,192 to 1:16,384 and anti-B 1:8,192 to 1:16,384. Although there was no constant relationship between single serum protein concentrations and iso-agglutinin titers, nevertheless there appeared to be a fairly well maintained rise in iso-agglutinin titers when the serum protein concentration was maintained at levels above 5.70 Gm. per cent. In the case of patient H. W. (Table IV) there was no correlation between the serum protein concentration and iso-agglutinin titers; in fact, the lowest titers (1:16 and 1:32) were found with normal serum protein levels. Patient H. S. (Table V) had serum protein concentrations which were consistently low and which varied between 3.93 and 4.11 Gm. per cent. Anti-A and anti-B titers showed variations of only three dilutions—1:128 to 1:1,024. Minor elevations in serum protein concentrations had no constant effect on iso-agglutinin titers.

TABLE IV

PATIENT H. W.
TYPE A

DATE (1943)	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
3/27	5.00		1:128
4/20	6.07		1:16
4/27	6.00		1:16
5/5	5.49		1:128
5/13	5.66		1:512
5/18	5.80		1:512
5/20	6.00		1:256
5/25	5.73		1:256
6/1	5.38		1:32
6/10	5.84		1:64
6/18	6.80		1:32
6/25	5.31		1:512
7/9	5.38		1:512
7/21	5.80		

TABLE V

PATIENT H. S.
TYPE O

DATE (1943)	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
4/13	3.90	1:512	1:512
4/21	4.05		
4/28	4.08	1:128	1:128
5/5	3.93	1:512	1:512
5/14	3.98	1:512	1:512
5/18	4.11	1:1024	1:256

DISCUSSION

Although the iso-agglutinin titers of the normal subjects included in this study were consistently higher than those observed by other investigators, we believe that these differences may be explained by the sensitive titration method which we employed and which included centrifugation and microscopic examination of the resuspended red cells. The range of dilutions in this normal group is not significantly different, however, from those recorded by other workers who employed different methods and obtained lower titers.

Low iso-agglutinin titers have been noted in severe anemias. Of the two patients with aplastic anemia, one (H. W.) had low titers; the other patient (A. W.) had iso-agglutinin titers within the normal range for the method used. No correlation was found in these two patients between red blood cell count, hemoglobin concentration, or total protein concentration and iso-agglutinin titers. The stored whole blood which was used for transfusion contained varying amounts of red cell stroma, but inasmuch as all blood was type-specific, it is unlikely that any elevation of iso-agglutinin titer can be attributed to this

source. Furthermore, no striking elevations in iso-agglutinin titers were observed immediately after transfusion, which excludes passively transferred iso-agglutinins as a factor responsible for the variations noted.

None of the subjects in our study had any of the diseases in which hyperglobulinemia was found by Kagan.[8]

With the exception of the case of A. W. (Table III), wherein a fairly well-maintained rise in iso-agglutinin titers accompanied the higher serum protein concentrations, that is, over 5.70 Gm. per cent, no constant relationship was observed between iso-agglutinin titers and total serum protein concentrations. We are unable to explain these observations.

SUMMARY AND CONCLUSIONS

1. Serum iso-agglutinin titers and total serum protein concentrations were determined simultaneously in normal subjects and those with hypoproteinemia. In three cases iso-agglutinin titers were determined during changes in serum protein concentration.

2. No constant relationship was found between blood iso-agglutinin titers and total serum protein concentration.

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A STUDY OF STAPHYLOCOCCUS ANTITOXIN TITERS IN NORMAL HUMAN SERA

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WITH the frequent therapeutic use of specific antitoxin in acute staphylococcus infections and of toxoid in those cases of chronic manifestations, the laboratory is being called upon more and more for determinations of the quantity of staphylococcus antitoxin in the patient's blood serum. To interpret laboratory examinations of any kind, it is necessary to know the results of the test in normal individuals and to evaluate the pathologic sample accordingly. Although an approximate normal value for circulating staphylococcus antitoxin has been known for some time, we felt that it would be expedient and worth while to test a large group of sera to establish an average normal and also a normal titer range. The purpose of this paper is to present the background, the technique, and the results of this problem.

Early reports of staphylococcal antihemolysin content are not readily comparable because of differences of standards or units employed in the various laboratories. The earliest reports in this country of titrations done to estimate staphylococcus antitoxin are those of Bryce and Burnet in 1932.[1] They did a series of normal titers on mothers and newborn infants, reporting a wide variation in titer; different individuals showing as little as 2 units or as much as 650 units, with the majority showing 40 to 160 units. The technique did not involve the use of international units and simply used an arbitrary "unit" value. The authors admitted a technical error in the titer readings of minus or plus 20 per cent. Their results showed that the titers of the mothers and the respective newborn infants were closely similar at birth; the infant's titer decreased up to the second year when it resumed the adult level where it remained until the sixth decade when a slight fall occurred.

In 1934, Parish, O'Meara, and Clark[2] described an antihemolysin titer technique by which 100 rabbits, 100 human beings, 50 horses, and 50 guinea pigs were tested. Since no standard antitoxin had been adopted, they chose to report their data in decimal fractions of a known antitoxin, which could later be linked with an official standard. Their results were as follows:

100 Rabbits	Titers from .003 to .03 of known K antiserum
100 Human beings	Titers from .003 to .03 of known K antiserum
50 Guinea pigs	Titers from .003 to .03 of known K antiserum
50 Horses	Titers from .003 to .07 of known K antiserum

McKie,[3] in 1934, reported 36 cases of superficial staphylococcus infections on which antihemolysin titers were determined. No mention was made, however, of the technique or "units" employed.

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Received for publication, June 26, 1944.

At this point it was obviously necessary to eliminate confusion in technique and terminology and to place antitoxin titrations on a much sounder basis. In 1935, the Health Organization of the League of Nations[4] reported in their Quarterly Bulletin a provisional stable standard for staphylococcus antitoxin. They defined 1 unit as the specific neutralizing activity contained in 0.5 mg. of the dry standard preparation prepared by the National Institute of Medical Research, London. When the stock of the above standard was depleted and revision was necessary, the Standardization Committee again chose an international standard antitoxin prepared by Wellcome Laboratories, with a mean potency of 412 international units per cubic centimeter. Using the international unit as the yardstick for reports, Murray,[5] in 1935, in studies of toxoid therapy found that a few normal controls ran a titer of 0.4 to 2 units; titers of 50 pregnant women gave an average reading of 0.75 unit; 50 newborn infants gave an average titer of 0.78 unit. In 1935, 14 normal sera were found by Blair and Hallman[9] to contain 1 unit or less; 46 Wassermann sera showed 1 unit or less. Whitby,[6] in 1936, examined one hundred normal sera which showed titers from 0.5 to 2 international units. In the study of natural staphylococcal immunity in monkeys, Weiss,[7] in 1939, found that the titers in 65 normal monkeys ranged from 0 to 25 international units; the majority were between 2 and 8 units. During a study of staphylococcal immunity in diabetic children, Bates and Weiss,[8] in 1941, reported titers found in normal children as follows: out of 14 children, 7 had titers of 0.6 unit or less, 1 had a titer of 1.0 unit, and 6 showed titers of 1.5 units.

METHODS

In the present study, tests were done on the blood sera of 500 adult males between the ages of 21 and 31 years. They were serologically negative draftees obtained through the local Selective Service Boards. Of these 500, 98 per cent passed the preliminary physical examination of the Army. The sera were inactivated at 56° C. for thirty minutes, and only clear unhemolyzed samples were used. The same series of Lederle's standard antitoxin and toxin were used throughout the tests; the antitoxin contained 42 units per cubic centimeter and the toxin was used in dilutions of 1:40 to 1:30 according to daily titrations.*

Levine[10] in 1939 showed conclusive evidence that all three properties of a staphylococcus toxin (lethal, necrotic and hemolytic) are due to the same toxic molecule and the corresponding antitoxin protects with identical results, whether tested for antihemolytic, antinecrotic, or antilethal properties. Therefore, three methods of testing the potencies of staphylococcus toxin-antitoxin mixtures are in use at the present (1) hemolytic, (2) dermonecrotic, and (3) lethal. A hemolytic test was used to determine the antitoxin titer in our series of 500 blood sera.

The unknown serum was run against a toxin dilution which had previously been found to produce 2 plus hemolysis against one unit of standard antitoxin. This dilution was determined by preliminary test each time unknowns were run, and the toxin adjusted to the proper dilution to produce 2 plus hemolysis. This preliminary titration eliminated inconsistencies which might be due to variations in the daily cell suspensions or the unstable toxin.

*The standard reagents were obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, New York.

A healthy rabbit was bled from an ear vein for 1 to 2 c.c. of blood which was oxalated or citrated. The cells were washed three times with physiologic saline and a final 1 per cent cell suspension made in saline. The standard antitoxin was diluted so that 1 unit was added to each tube, or, as in testing unknown human sera of low potency, 1/8 unit of antitoxin was employed instead of the 1 unit. Our Lederle LF serum No. 6 contained 42 units per cubic centimeter; therefore, it was diluted 1:168 to obtain 1/8 unit in 0.5 c.c. dilution. This antitoxin dilution was kept in the refrigerator and used one week before a fresh dilution was prepared. In the preparation of the preliminary toxin titration, dilutions of the toxin were set up in five or six serial dilutions within the range of its potency. These dilutions were prepared fresh each time. An equal amount of each of these dilutions was added to a series of tubes containing the 1/8 unit of antitoxin. A doubled amount of 1 per cent washed rabbit cells was added and the titration incubated at 37° C. for one hour. Two controls were included to insure the fact that neither the saline nor the antitoxin dilution were hemolytic. The dilution tube showing 2 plus hemolysis contained the correct amount of toxin to be balanced and combined by the 1/8 unit of antitoxin present.

TABLE I

NUMBER OF SERA	PER CENT	INTERNATIONAL UNITS OF STAPHYLOCOCCUS ANTITOXIN
17	3.4	0
65	13.0	0.25
46	9.2	0.375
119	23.8	0.5
103	20.6	0.75
85	17.0	1.0
40	8.0	1.5
12	2.4	2.0
9	1.8	3.0
4	0.8	4.0
Total number of sera tested—500 Normal serum average—0.75 international unit		

As expressed in units the average titer of normal serum was 0.75 international unit.
The majority, or 61 per cent, of the sera contained 0.5 unit, 0.75 unit, and 1.0 unit.
150 sera, or 30 per cent, contained titers above the average figures.
247 sera, or 49 per cent, contained titers below the average figure of 0.75 unit.
65 sera, or 13 per cent showed antitoxin content higher than 1 unit per cubic centimeter.
17 sera, or 3.4 per cent contained no demonstrable antitoxin.

In testing an unknown blood serum, a series of dilutions was prepared ranging from an undiluted sample to a 1:64 dilution. An equal quantity of the previously determined toxin dilution was added, followed by a doubled amount of rabbit cells. One control tube was included containing 1/8 unit of standard antitoxin plus equal amount of toxin dilution plus red cells; it would read as 2 plus hemolysis if a proper balance was obtained for the standard antitoxin and toxin and cells.

The tubes were heated for one hour in a water bath at 37° C. The end point was at 2 plus hemolysis as compared with the 2 plus control. This end point gave the dilution titer of the serum, which was converted to international units per cubic centimeter by dividing the dilution by 4. This calculation is

based upon the fact that ⅛ unit of antitoxin in 0.5 c.c. was used for standard, or the equivalent of ¼ unit in 1.0 c.c. Therefore the dilution was multiplied by ¼ or divided by 4.

RESULTS

In our group of 500 sera, only 10 or 20 were titered at one time, because it was felt that accuracy was sacrificed when larger numbers were handled. If the end point appeared to be between adjacent dilutions, e.g., 1:2 and 1:4, the titer was read between the two, as 1:3. When the unit reading was below 1 unit, it was expressed in decimal fractions of 2 places, e.g., 1:3 dilution divided by 4 to give a 0.75 unit value.

Table I gives the results of the titer determinations, showing the number of sera with their respective international unit value.

DISCUSSION

In considering the use and relationship of antitoxin titers for diagnosis and therapy, the literature shows many studies on both animals and man.

Weiss[11] states that the rate of absorption, as measured by the blood serum content of antitoxin after intramuscular injection of antitoxin, is uniform in both monkeys and man. The amount of injected antitoxin corresponds uniformly with the serum determinations in cases using both small and large amounts of antitoxin.

The localization and concentration of antitoxin[12] in artificially produced areas of inflammation is significant and the use of counterirritants in the treatment of local infections is therefore indicated.

The resistance of rabbits to the intravenous injection of staphylococci suspensions is definitely correlated with the antitoxin content of their serum.[13] Increased antitoxin titers decreased mortality rates from 80 per cent in normal titered rabbits to 9 per cent in antitoxin-treated ones. Parish and Clark[15] agree with these findings which certainly seem to justify more extensive human clinical use of antitoxin.

With histologic studies on mice immunized both actively and passively and then inoculated with staphylococci, the mechanism of immunity with antitoxin appears primarily as neutralization of the toxin.[14] Leucocytes survive for longer periods in immune animals and continue to phagocytize the staphylococci more easily in presence of immune serum.

On human volunteers and patients with staphylococcic infections there has been considerable experimentation, employing staphylococcal toxoid to produce active immunization. Dolman,[17] McKie,[3] Murray,[5] Blair and Hallman,[22] Whitby,[6] Buchman,[16] Longacre,[23] and others have contributed in this field. In thirty-six cases of superficial staphylococcic infections, a rise in antihemolysin titer is accompanied by a clinical improvement.[3]

Amounts of antitoxin are not markedly different in normal persons and in those with chronic superficial lesions like furunculosis. There are varying results reported by different workers on the significance of diagnosis and therapy in chronic osteomyelitis. It is suggested that strains of staphylococci causing

these deep types of infections are of low toxigenicity and of high invasiveness, hence there is little correlation between the antitoxin titer and the progress of the disease.

In another series,[6] toxoid therapy was found to be clinically effective in furunculosis, styes, carbuncles, but not in cases of pustular acne and sycosis.

An interesting comparison of antitoxin production in diabetic and normal children,[8] showed delayed production and lowered maximum titers in diabetic children. There was also a correlation between the state of diabetic controls and antitoxin response: well-controlled diabetic cases responded similarly to normal children, and poorly controlled cases showed retardation and lowered production.

The value of passive immunity produced by the injection of antitoxin has been studied as a therapeutic agent. In 104 cases of staphylococcus infections treated with antitoxin,[17] 24 out of 24 recovered from skin and subcutaneous infections; 22 out of 32 cases of staphylococcemia from osteomyelitis recovered; 5 out of 22 hopeless staphylococcemia in adults recovered. Other cases,[18-20] of staphylococcemia, and acute osteomyelitis showing favorable response to antitoxin therapy were cited. The fatality of staphylococcemia in 117 cases was found to be 91.4 per cent.[21] In a study of 17 cases of the same type treated with staphylococcal antitoxin, Stookey and Scarpellino reported a 53 per cent recovery. Early diagnosis and administration of antitoxin before the toxin gets fixed to the tissues are distinct aids in lowering the mortality rate.

The consensus of these various authors is that, when there is adequate drainage of pyogenic foci, staphylococcus antitoxin is a specific therapeutic agent of considerable usefulness. There is no contraindication to its use with other forms of therapy, such as blood transfusions, sulfonamides, or bacteriophage.

SUMMARY

A technique for determining staphylococcus antitoxin titers in blood serum by a hemolytic test has been described.

An average normal titer has been calculated from a series of 500 normal blood samples.

Reports are cited from the literature which indicate the value of direct antitoxin therapy or toxoid administration.

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STUDIES ON THE TOXICITY OF GOLD COMPOUNDS IN RATS

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THE reports of Møllgaard,[1] Forestier,[2] Towle and Grund[3] Hartfall, Garland, and Goldie,[4] Sabin and Warren,[5, 6] and others have created considerable interest in the use of gold compounds for the treatment of tuberculosis, lupus erythematosus, and arthritis. However, the frequent, varied toxic reactions and occasional fatalities in patients receiving gold drugs have been a hindrance to their extensive use. In an effort to find a nontoxic, therapeutically effective drug, investigators have tested a large number of gold preparations without discovering one that is completely satisfactory.

In 1940 Sabin and Warren[6] studied the effectiveness of gold compounds in curing an experimental mouse arthritis produced by inoculating mice with a pleuropneumonia-like organism. In their work they studied the toxicity of several gold compounds, using mice as test animals and using survival or death of the animal as an index of toxicity. Obviously this method gives no measure of the degree of toxicity in surviving animals.

In using a similar technique to test the toxicity of gold compounds in this laboratory, it was noted that mice receiving sublethal doses of gold salts exhibited an elevation of the nonprotein nitrogen level of the blood, often to two or three times normal values. From this it appeared that the nonprotein nitrogen value of the blood might be a better index of toxicity than survival or death. However, since a mouse had to be sacrificed to obtain sufficient blood for a nonprotein nitrogen determination, this method could hardly be considered satisfactory since it is often desirable to give an animal several doses of the drug. In order to eliminate the necessity of sacrificing an animal whenever its blood was to be analyzed, rats were used in the experiments reported here.

In the following report several gold compounds, some commercially available and others especially synthesized for this work, have been studied in order to obtain information with regard to their toxic reactions.

GOLD COMPOUNDS USED

Table I lists the gold compounds used in the following experiments together with their formulas and theoretical gold content. Especially prepared gold

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This paper is taken from a thesis presented to the Graduate School of the Pennsylvania State College by Charles W. Denko in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Authorized for publication July 18, 1944, as paper No. 1239 in the journal series of the Pennsylvania Agricultural Experiment Station.

Received for publication, Aug. 5, 1944.

compounds were analyzed for gold as an index of purity. These values are given together with the method used for the preparation of each prepared compound.

TABLE I

GOLD COMPOUNDS USED

NAME	FORMULA	PER CENT OF GOLD		METHOD OF PREPARATION
		THEORETICAL	FOUND§	
1. Aurothioglucose*	$C_6H_{11}O_6SAu$	50.		
2. Gold sodium thiosulfate†	$Na_3Au(S_2O_3)_2 \cdot 2H_2O$	37.4		
3. Colloidal gold sol‡				
4. Strontium aurothioglycolate	$Sr(OOCH_2SAu)_2$	59.56	60.61	(7), (8)
5. α-aurothio-*n*-butyric acid	$CH_3(CH_2)CH(SAu)COOH$	62.24	61.40	(7), (8)
6. Auro-*n*-propyl xanthate	$CH_3(CH_2)_2OCSSAu$	59.29	59.34	(9), (10)
7. Auro-isopropyl xanthate	$(CH_3)_2CHOCSSAu$	59.29	57.70	(9), (10)
8. Auro-*n*-butyl xanthate	$CH_3(CH_2)_3OCSSAu$	56.92	55.73	(9), (10)
9. Auro-isobutyl xanthate	$(CH_3)_2CHCH_2OCSSAu$	56.92	56.64	(9), (10)

*Supplied through the courtesy of the Schering Corporation, Bloomfield, N. J.
†Supplied through the courtesy of Mr. J. G. Bieri.
‡Supplied through the courtesy of Crookes Laboratories, Inc., New York, N. Y.
§Method of the American Medical Association.[11]

TOXICITY OF GOLD COMPOUNDS

The effects of gold compounds on the nonprotein nitrogen of the blood are given in Table II. The Anderson-Howell[12] method was used to determine all the nonprotein nitrogen values. Gold sodium thiosulfate was administered in water solution. The colloidal gold was a hydrosol. Water-insoluble strontium aurothioglycolate and the auroxanthates were administered intramuscularly in a sesame oil suspension. Since α-aurothio-*n*-butyric acid is soluble only to the extent of 54 mg. per 100 c.c. of water at 100° C., it also was administered in oil suspension. Gold sodium thiosulfate was injected as an aqueous solution.

Gold sodium thiosulfate is definitely toxic when administered in 5 mg. doses. In Animal 23 death occurred on the third day when the blood nonprotein nitrogen had reached a level of 248.1 mg. per 100 c.c. In Animal 24 the blood nonprotein nitrogen reached a level of 151 mg. on the fourth day but returned to normal on the seventh day. In animals receiving less than 5 mg. there was no appreciable rise in blood nonprotein nitrogen except in Animal 28, receiving 2 mg., where a value of 150.1 mg. was found on the second day after injection. This animal returned to normal on the third day.

α-Aurothio-*n*-butyric acid was toxic when administered at a 3 mg. level, one animal having a nonprotein nitrogen level of 339 mg. and dying the third day after injection. On the 5 mg. level one animal died the third day when the nonprotein nitrogen level had reached 185.4 mg. per 100 c.c. of blood. The three other animals receiving 5 mg. doses had high nonprotein nitrogen values the third day after injection. The fifth day after injection two of these were essentially normal, while the third had returned to normal the tenth day.

In the case of all the other compounds studied, there was no increase in nonprotein nitrogen level even after doses of 100 mg. in the cases of auro-*n*-butyl and auro-isobutyl xanthates.

TABLE II

SHOWING NONPROTEIN NITROGEN OF RAT BLOOD BEFORE AND AFTER INJECTING GOLD PREPARATIONS

ANIMAL	SEX	WEIGHT (GM.)	DOSE (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)											
				BEFORE INJECTION	DAYS AFTER INJECTION										
					1	2	3	4	5	7	8	10	11	12	13
Gold sodium thiosulfate															
30	F	106	1	54.6	52.8	44.4	44.1			36.9					
29	F	139	1	39.1	37.1	46.2	36.6			43.2					
28	F	139	2	39.6	41.4	150.1	42.7			41.4					
27	M	167	2	39.4	46.5	45.6	53.2			48.6					
26	F	121	3	40.6	48.2	52.2	39.2			43.9					
25	F	113	3	41.0	42.8	44.1	45.3			45.3					
24	M	157	5	69.7	85.4	139.6	149.4	151.0		42.2					
23	M	114	5	43.9	136.4	156.2	248.1*								
α-aurothio-*n*-butyric acid															
35	F	169	3	42.4	96.3	175.6	339.0*								
32	M	185	3	38.1	35.5	34.3	47.4		37.9						
37	M	146	5	51.9	90.6	151.2	180.9		56.4			39.7			
36	F	167	5	51.1	95.9	92.9	131.6		37.3			43.9			
33	F	185	5	42.6	96.4	151.5	170.4		192.3			42.1			
31	M	211	5	40.4	131.7	176.4	185.4*								
Colloidal gold (Ruby)															
38	M	201	1.25	64.2	39.3	50.7		39.1							
39	F	177	2.25	42.4	36.3	37.8		41.2							
Strontium aurothioglycolate															
40	M	215	5	47.4	36.1	40.4	40.4								
41	M	202	14	33.4	43.0	39.9	41.4								
Auro-*n*-propyl xanthate															
44	F	141	5	35.9	35.9	35.6	34.0								
45	M	144	10	35.1	30.7	30.5	38.9								
46	M	241	15	46.4	33.6	48.3	34.4								
47	M	217	25	37.5	41.1	41.1	33.7								
Auro-isopropyl xanthate															
57	F	141	5	42.0	35.5	35.4	38.1	37.8							
56	M	233	10	36.7	39.3	47.1	36.1	43.9							
58	F	186	15	39.1	31.2	40.0	37.5	44.6							
59	M	238	25	37.1	40.7	40.4	40.4	37.4							
Auro-*n*-butyl xanthate															
48	M	174	10	41.6	30.7	37.0		36.9							
49	M	192	20	31.0	38.6	44.0		35.3							
50	F	190	50	40.8	40.0	40.3		51.5							
51	F	189	100	51.0	36.3	39.6		41.7					38.5	35.3	49.7
Auro-isobutyl xanthate															
52	F	203	10	40.5	34.0	36.3	42.9			46.5					
53	M	245	20	42.3	39.7	36.0	39.7	35.7		41.3					
54	F	183	50	30.7	30.7	35.6	38.5	43.2		38.3	36.8				
55	M	240	100	31.5	31.0	36.9	36.3	34.9							

*Died.

The results of this experiment indicate that the toxicity of gold compounds depends upon their solubility in water. Gold sodium thiosulfate and α-aurothio-*n*-butyric acid, which are water soluble, are toxic, whereas colloidal gold, strontium aurothioglycolate, and the auroxanthates, water-insoluble substances, are nontoxic, as indicated by their effects on the nonprotein nitrogen values

of the blood. If a gold compound is toxic and the animal survives the dose given, the toxic reaction is temporary, the nonprotein nitrogen value of the blood returning to normal in from five to ten days after injection.

THE EFFECT OF TYPE OF CARRIER ON THE TOXICITY OF AUROTHIOGLUCOSE

Some of the compounds in the previous experiments were administered in oil suspensions and some were administered in aqueous solution. Many workers feel that administration of water-soluble aurothioglucose as an oil suspension makes this compound less toxic than when administered in aqueous solution. In order to determine the effect of type of carrier on the toxicity of aurothioglucose, animals were given intramuscular injections of varying doses in oil suspension and in aqueous solution. The effects of these injections on the nonprotein nitrogen level of the blood are given in Table III.

TABLE III

SHOWING THE EFFECT OF TYPE CARRIER ON THE TOXICITY OF AUROTHIOGLUCOSE

ANIMAL	SEX	WEIGHT (GM.)	DOSE (MG.)	CARRIER	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)						
					BEFORE INJECTION	DAYS AFTER INJECTION					
						1	2	3	4	7	15
1	F	141	Control 1.0 c.c.	0.9% NaCl	53.3	43.5	50.7				34.6
10	M	185	Control 1.0 c.c.	Sesame oil		51.8	41.2	39.8		44.9	
2	M	161	10	Water	36.4	54.7	40.7	40.7		42.9	44.4
6	F	134	10	Oil	37.7	61.1	44.3	50.2		38.1	
4	F	143	40	Water	45.3	141.0	169.5	184.1		87.8	44.6
7	F	162	25	Oil	55.0	131.0	176.5	266.8*			
3	F	170	50	Water	51.8	101.0	197.5	151.5		81.2	44.2
8	M	180	50	Oil	60.0	126.0	214.2	205.6	363.6*		
5	M	184	100	Water	48.6	97.8	268.0	287.2	340.0*		
9	M	169	100	Oil	54.3	120.0	238.0	309.0		107.5	

*Died.

Table III shows a comparison of the effect of various doses or aurothioglucose on the nonprotein nitrogen level of the blood when administered in water solution and in oil suspension. There appears to be little difference in the toxicity of aurothioglucose as related to the mode of injection. On the 25 and 50 mg. levels, aurothioglucose appears to be more toxic when administered in oil than when administered in water solution, as indicated by the death of both animals receiving the oil suspension. On the 40 and 50 mg. levels in water solution both animals survived and were back to normal the fifteenth day. However, on the 100 mg. level death occurred in the animal receiving the water solution, whereas the animal receiving the oil suspension survived.

TOXICITY OF α-AUROTHIO-*n*-BUTYRIC ACID, DUE TO GOLD ATOM

In order to prove that the toxicity of gold compounds was due to the presence of gold, animals were given injections of α-thio-*n*-butyric acid and later α-aurothio-*n*-butyric acid. The effects of these compounds on the nonprotein nitrogen level of the blood are given in Table IV.

TABLE IV

TOXICITY OF α-AUROTHIO-*n*-BUTYRIC ACID DUE TO PRESENCE OF GOLD ATOM

ANIMAL	SEX	WEIGHT (GM.)	α-THIO-*n* BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)				α-AUROTHIO-*n*-BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)				
				BEFORE INJECTION	DAYS AFTER INJECTION				DAYS AFTER INJECTION				
					1	2	3		1	2	3	5	10
31	M	201	50	36.9	41.3	47.8	40.4	5	131.7	176.4	185.4*		
32	M	181	25	48.4	41.4	38.1		3	35.5	34.3	47.4	37.9	
33	F	174	10	47.2	37.9	39.7	42.6	5	96.4	151.5	170.4	192.3	42.1
34	F	174	0.50 c.c. 0.9% NaCl	48.0	45.4	37.8	39.7	0.50 c.c. oil	41.4	42.3	42.3	43.9	

*Died.

Table IV indicates that α-thio-*n*-butyric acid, when administered in doses up to 50 mg., is nontoxic. When this is followed in the same animals by doses of 5 mg. of α-aurothio-*n*-butyric acid, definite toxicity results as indicated by a rise in blood nonprotein nitrogen values, and in one case by the death of the animal. It is evident from this that toxicity is due to gold.

DEVELOPMENT OF TOLERANCE TO AUROTHIOGLUCOSE AND GOLD SODIUM THIOSULFATE

Cortell and Richards [13, 14] studied the development of tolerance to gold salts in rats, using the survival method. They showed that rats given sublethal doses of gold sodium thiosulfate and of gold sodium thiomalate could tolerate ordinarily lethal doses of these compounds subsequently administered. They also showed that a rat which had developed tolerance to gold sodium thiomalate was also tolerant to gold sodium thiosulfate.

The results presented in Table V show the effects of repeated doses of aurothioglucose and of gold sodium thiosulfate on blood nonprotein nitrogen values. Rats given doses of 40 and 50 mg. of aurothioglucose showed a decided rise in blood nonprotein nitrogen values which returned to normal the fifteenth day. When the doses were repeated on these animals, blood nonprotein nitrogen values remained normal. A third dose of 75 and 100 mg. also showed no appreciable rise in the blood nonprotein nitrogen value. Animal 2, given an initial dose of 10 mg., a second dose of 20 mg., and a final dose of 50 mg., showed little change in blood nonprotein nitrogen values.

The results obtained with gold sodium thiosulfate although similar to those obtained with aurothioglucose are not as consistent. Seven rats were given initial doses ranging from 1 to 5 mg. Only two animals showed a rise in blood nonprotein nitrogen values, the one receiving 2 mg. and the other 5 mg. As a second dose each animal was given 5 mg. Apparently an initial dose of 1 mg. did not protect the animal since both of these animals died after the 5 mg. dose. Even initial doses of 2 and 3 mg. showed slight protection since in all cases the blood nonprotein nitrogen values were high, and in Animal 26, which had received an initial dose of 3 mg., death occurred. An initial dose of 5 mg. apparently protected the animal because there was little rise in the blood nonprotein nitrogen value after the second dose of 5 mg.

The four surviving animals were each finally given a 10 mg. dose. All animals survived and blood nonprotein nitrogen values were approximately normal on the seventh day after injection except in Animal 28. Animal 28

TABLE V

SHOWING DEVELOPMENT OF TOLERANCE TO AUROTHIOGLUCOSE AND GOLD SODIUM THIOSULFATE

ANIMAL	SEX	WEIGHT GM.		NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)						
				BEFORE INJECTION	DAYS AFTER INJECTION					
					1	2	3	4	7	9	15
AUROTHIOGLUCOSE											
			1st Dose mg.								
1	F	141	1 c.c. NaCl	53.3	43.5	50.7					34.6
2	M	161	10	36.4	54.7	40.7	40.7		42.9		44.4
3	F	143	40	45.3	141.0	169.5	184.1		87.8		44.6
4	F	170	50	51.8	101.0	197.5	151.5		81.2		44.2
			2nd Dose mg.								
1		150	0.5 c.c. NaCl		60.8	49.4	51.1			56.4	
2		212	20		47.1	41.7	53.0			52.4	
3		155	40		49.8	39.1	44.2			49.9	
4		175	50		44.7	42.2	48.4			53.8	
			3rd Dose mg.								
1		168	1 c.c. NaCl		48.7	50.7					
2		221	50		51.9	44.8	54.6				
3		159	75		48.9	41.6	47.5				
4		187	100		50.7	47.4	52.4				
GOLD SODIUM THIOSULFATE											
			1st Dose mg.								
29	F	139	1	39.1	37.1	46.2	36.6		43.2		
30	F	106	1	54.6	52.8	44.4	44.1		36.9		
27	M	167	2	39.4	46.5	45.6	53.2		48.6		
28	F	139	2	39.6	41.4	150.1	42.7		41.4		
25	F	113	3	41.0	42.8	44.1	45.2		45.3		
26	F	121	3	40.6	48.2	52.2	39.2		43.9		
24	M	157	5	69.7	85.4	139.6	149.4		42.2		
			2nd Dose mg.								
29		149	5		150.0	207.2	280.0*				
30		126	5		94.2	174.0	240.0*				
27		167	5		123.6	42.4	54.5		49.4		
28		139	5		104.8	116.2	153.8		100.0		
25		133	5		117.8	119.5	100.0		84.8		
26		141	5		86.0	209.0	175.6	255.6*			
24		157	5		78.3	47.1	39.4		49.8		
			3rd Dose mg.								
27		214	10		92.7	78.9	63.1		55.7		
28		146	10		137.4	197.8	255.4		159.6		
25		138	10		162.4	95.0	63.1		48.1		
24		193	10		105.2	132.2	85.0		44.1		

*Died.

appears to have been exceptionally sensitive to gold since the initial dose of 2 mg. produced an appreciable rise in the blood nonprotein nitrogen value.

The results of these experiments indicate that rats become more tolerant to gold compounds after having received sufficiently large initial doses of them.

DEVELOPMENT OF CROSS-TOLERANCE

Animals that had received aurothioglucose were injected with gold sodium thiosulfate and later with α-aurothio-*n*-butyric acid. Animals that had re-

ceived gold sodium thiosulfate were injected with aurothioglucose and later with α-aurothio-*n*-butyric acid. Animals that had received auroxanthates were subsequently injected with aurothioglucose. The changes in the nonprotein nitrogen values of the blood following these injections are presented in Table VI.

TABLE VI

CROSS-TOLERANCE STUDIES

ANIMAL	SEX	WEIGHT (GM.)	GOLD SODIUM THIOSULFATE (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)						α-AUROTHIO-*n*-BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)			
				BEFORE INJECTION	DAYS AFTER INJECTION						DAYS AFTER INJECTION			
					1	2	3	5	9		1	2	3	7
Aurothioglucose*-gold sodium thiosulfate-α aurothio-*n*-butyric acid														
3	F	181	10	52.4	98.0	76.9	49.6		42.7	10	62.1	41.8	44.6	40.0
Gold sodium thiosulfate†-aurothioglucose-α-aurothio-*n*-butyric acid														
			Aurothioglucose (mg.)											
27	M	183	100	55.7		49.9	87.3	50.0		10	61.1	55.8	43.1	
Auro-*n*-propyl xanthate†-aurothioglucose														
45	M	158	100	34.0	38.2	42.1	39.9	42.8						
Auro-*n*-butyl xanthate†-aurothioglucose														
49	M	214	50	35.3	50.6	51.7	56.6							
Auro-isobutyl xanthate†-aurothioglucose														
52	F	213	100	46.5	49.3	58.3								
Auro-isopropyl xanthate†-aurothioglucose														
58	F	179	50	44.6	77.0	112.8	125.0							
Controls—aurothioglucose														
64	M	186	50	49.4	102.3	132.4								
62	M	257	100	39.3	164.8	170.5								

*See Table II for effects of original doses.
†See Table I for effects of original doses.

Animal 3, which had originally received a dose of 50 mg. of aurothioglucose and whose blood nonprotein nitrogen value had reached 197.5 mg. per cent two days after injection, tolerated a 10 mg. dose of gold sodium thiosulfate. The blood nonprotein nitrogen value reached 98.0 mg. per cent one day after the gold sodium thiosulfate was given. Later this animal tolerated a 10 mg. dose of α-aurothio-*n*-butyric acid with very little rise in the blood nonprotein nitrogen value. Ten milligram doses of gold sodium thiosulfate and α-auro-*n*-butyric acid given to a normal rat are usually lethal.

Animal 27 originally received 2 mg. of gold sodium thiosulfate with little subsequent rise in blood nonprotein nitrogen values. When later given 100 mg. of aurothioglucose, the blood nonprotein nitrogen value rose to 87.3 mg. per cent three days after injection. Later this animal tolerated 10 mg. of α-aurothio-*n*-butyric acid with little rise in blood nonprotein nitrogen values.

As indicated in Table II, auroxanthates, even when given in 100 mg. doses, showed no toxicity as indicated by blood nonprotein nitrogen values. However, animals which had received auroxanthates show tolerance for subsequently administered aurothioglucose, as indicated in Table VI. Whereas

50 and 100 mg. doses of aurothioglucose show marked rises of blood nonprotein nitrogen values when given to normal animals, very little rise in blood nonprotein nitrogen values is noted when these doses are given to animals previously given auroxanthates. Of the auroxanthates studied, auro-isopropyl xanthate appears to be the only one that did not protect the animal against the toxic effect of subsequently administered aurothioglucose. Although the therapeutic value of auroxanthates has not been demonstrated, their effect in desensitizing animals is clearly demonstrated.

HISTOPATHOLOGIC STUDIES

Cortell and Richards[14] have found severe kidney damage in rats receiving gold sodium thiosulfate. Brown, Saleeby, and Schamberg,[15] in a study on rabbits, reported that marked injury to the kidneys may take place before definite blood changes occur. Block, Buchanan, and Freyberg[16, 17] have reported finding gold in the kidneys and other organs of the body following administration of various gold drugs.

In the studies here reported, autopsies were performed on all animals. Histopathologic studies of the kidney were made on several representative animals by Dr. W. T. S. Thorp, of the Animal Pathology Laboratory.

Insoluble gold compounds were found largely unabsorbed at the site of injection for as long as two weeks after administration. No evidence of the presence of the soluble compounds was noted. Nephritis was noted in most cases studied histologically. In all cases of nephritis a high blood nonprotein nitrogen value was noted. However, kidney damage was not observed in all cases where a high nonprotein nitrogen value occurred. From this it would appear that blood nonprotein nitrogen values serve as a more sensitive index of toxicity than the histologic study of the kidney.

SUMMARY

1. The nonprotein nitrogen value of the blood has been used in determining the toxicities of several commercial and experimentally synthesized gold compounds.

2. The water-soluble compounds, gold sodium thiosulfate, aurothioglucose, and slightly soluble α-aurothio-*n*-butyric acid, were found to be toxic.

3. The water-insoluble compounds, colloidal gold and auro-*n*-propyl, auro-isopropyl, auro-*n*-butyl and auro-isobutyl xanthates, were found to be nontoxic.

4. The toxicity of α-aurothio-*n*-butyric acid was found to be due to the gold portion of the molecule.

5. Tolerance and cross-tolerance to gold compounds were developed in animals.

6. High blood nonprotein values were always found where tissue changes indicating kidney damage had occurred. In some cases high blood nonprotein nitrogen values occurred where there was no evidence of tissue change.

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THE ANTIBACTERIAL EFFECTS OF SEVERAL ACRIDINE COMPOUNDS

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THE antibacterial effects of a number of acridine derivatives against both gram-negative and gram-positive organisms were presented in two previous communications from these laboratories.[1, 2] The compounds tested included derivatives of Atabrine* [3-chloro-7-methoxy-9(1-methyl-4-diethylaminobutylamino)-acridine], Rivanol* (3-ethoxy-6, 9-diamino-acridine) and acranil [3-chloro-7-methoxy-9(2-hydroxy-3-diethylamino-propylamino)-acridine]. It was demonstrated that many of these compounds had an action comparable to the antibacterial effects *in vitro* of acriflavine and proflavine. The mode of action of the acridines was found to be largely bactericidal rather than merely bacteriostatic in the drug concentrations tested.

In view of the above findings it appeared worth while to extend these studies to include several other acridines which in preliminary tests showed promise as highly active bactericidal agents *in vitro*. These included the sulfamate salt of 9-aminoacridine, 3-chloro-7-methoxy-9-aminoacridine and 3-chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate (an Atabrine analogue). 9-Aminoacridine which was first prepared and tested by Albert et al.[3] in 1938 under the designation of 5-aminoacridine† and the hydrochloride of this compound completed our series. Ungar and Robinson[4] found the latter two compounds to be less toxic to tissues and interfered less with the healing of artificial wounds than either proflavine or acriflavine.

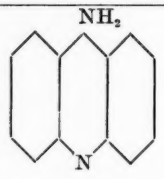
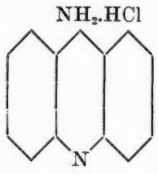
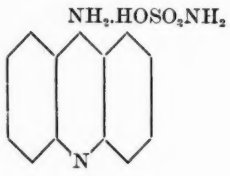
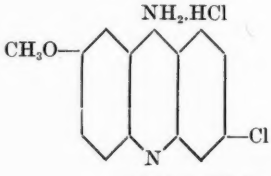
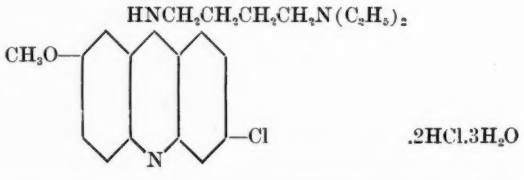
The organisms against which the compounds were tested included *Staphylococcus aureus* No. 209, beta hemolytic streptococcus No. C-203, *Streptococcus viridans, Streptococcus agalactiae,* Types I, II, and III pneumococci, *Bacillus pyocyaneus, Clostridium welchii, Clostridium tetani, Clostridium histolyticus,* and *Clostridium septicum.* The compounds tested together with their molecular weights and structural formulae are given in Table I.

METHODS

The method used in testing the several acridines was essentially the same as outlined in the previous report.[2] It consisted of preparing a 1:1,000 dilution of a compound in nutrient broth medium. Veal infusion broth containing 0.15 per cent dextrose was used in studying the pneumococci and streptococci; bacteria of the anaerobic Clostridium group were tested in Brewer's fluid thioglycollate medium[5]; beef infusion broth containing 0.15 per cent dextrose was used for the remaining organisms. Serial dilutions through 1:4,096,000 were made in the respective media. The tubes containing the drug dilutions and control broth tubes lacking the drug were autoclaved at 10 pounds for 10 min-

From the Research Laboratories, Winthrop Chemical Company, Inc.
Received for publication July 21, 1944.
*Trade-marks of Winthrop Chemical Company, Inc.
†Also known as Monacrine in Australia.

TABLE I

COMPOUND	MOLECULAR WEIGHT	STRUCTURAL FORMULA
9-Aminoacridine	194.2	
9-Aminoacridine hydrochloride	230.7	
9-Aminoacridine sulfamate	291.3	
3-Chloro-7-methoxy-9-aminoacridine hydrochloride	295.2	
3-Chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate	512.9	

utes. After cooling, normal sterile horse serum was added to the tubes containing the veal-dextrose broth to a concentration of 1 per cent.

Preliminary solubility studies showed 9-aminoacridine and 3-chloro-7-methoxy-9-aminoacridine hydrochloride to be but slightly soluble in water. These compounds were dissolved, therefore, in a few c.c. of ethyl alcohol preparatory to making the initial broth dilution. The greatest concentration in which the latter compound would remain in solution completely was 1:8,000.

A preliminary test on an aqueous solution of 3-chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate revealed that it lost approximately 50 per cent of its activity upon autoclaving. For this reason a 1 per cent aqueous solution of the compound was sterilized by Berkefeld filtration. Subsequent serial dilutions from the stock solution were prepared aseptically in sterile broth.

The highest drug concentration of all the acridines studied did not alter the original pH of the broth media. Suitable control demonstrated that the small

TABLE II

HIGHEST DILUTION OF COMPOUND EXHIBITING ANTIBACTERIAL ACTIVITY

	9-AMINO-ACRIDINE		9-AMINO-ACRIDINE HCl		9-AMINO-ACRIDINE SULFAMATE		3-CHLORO-7-METHOXY-9-AMINO-ACRIDINE HCl		3-CHLORO-7-METHOXY-9-(4-DIETHYLAMINOBUTYL-AMINO)-ACRIDINE $2HCl.3H_2O$	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
Pneumococcus Type I	256,000	256,000	256,000	128,000	128,000	128,000	256,000	256,000	256,000	128,000
Pneumococcus Type II	256,000	128,000	256,000	128,000	128,000	128,000	512,000	256,000	512,000	256,000
Pneumococcus Type III	128,000	128,000	16,000	2,000	16,000	4,000	256,000	128,000	512,000	256,000
Streptococcus Hemolyticus	128,000	128,000	128,000	128,000	128,000	32,000	128,000	128,000	256,000	64,000
Streptococcus Viridans	64,000	8,000	64,000	32,000	32,000	8,000	128,000	128,000	64,000	32,000
Streptococcus Agalactiae	128,000	128,000	128,000	128,000	128,000	32,000	128,000	64,000	128,000	16,000
Staphylococcus Aureus	64,000	8,000	32,000	8,000	32,000	8,000	64,000	<8,000	64,000	4,000
Bacillus pyocyaneus	64,000	2,000	64,000	2,000	64,000	1,000	<8,000	<8,000	1,000	<1,000
Clostridium Welchii	128,000	128,000	128,000	128,000	128,000	64,000	256,000	256,000	1,024,000	256,000
Clostridium Tetani	128,000	128,000	128,000	128,000	128,000	128,000	256,000	128,000	1,024,000	256,000
Clostridium histolyticus	256,000	128,000	256,000	32,000	128,000	32,000	512,000	8,000	2,048,000	32,000
Clostridium Septicum	256,000	128,000	128,000	128,000	128,000	128,000	512,000	256,000	2,048,000	512,000

Bs = Bacteriostatic.
Bc = Bactericidal.
<8,000 = This compound insoluble in concentrations greater than 1:8,000, hence could not be tested.
<1,000 = Concentrations greater than 1:1,000 not tested.

amount of alcohol used in dissolving some of the compounds had no inhibitory effect upon the growth of the test organisms.

Each tube of a dilution series was inoculated with a loopful of a 24-hour broth culture of one of the test organisms. The tubes were incubated at 37° C. and examined for visible growth after 24, 48, and 72 hours. Lack of growth after 24 hours was considered evidence of bacteriostasis. Tubes which failed to show growth after 72 hours were tested for bactericidal activity by transferring 3 loopfuls of the drug-organism-broth mixture to a broth medium lacking the drug. Failure of growth to appear in the subculture tube was taken as evidence of bactericidal action on the part of the drug in the original medication tube. The results of these studies are presented in Table II.

RESULTS

The high degree of antibacterial activity of the several compounds against the organisms tested is evident from the data presented in the above table. Although not shown in this table, the 9-aminoacridines and 3-chloro-7-methoxy-9-aminoacridine hydrochloride exhibit bacteriostatic and bactericidal properties similar to the most active acridines heretofore studied, namely, acriflavine and proflavine.[2]

In general 3-chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate compared in activity with the other acridines against the several gram-positive cocci. It was found, however, to be considerably less active than the latter preparations against the gram-negative organism, *Bacillus pyocyaneus*. Of significant interest is the relatively high degree of antibacterial action of this Atabrine analogue against the anaerobic bacteria. Dilutions of 1:1,000,000 to 1:2,000,000 proved to be bacteriostatic for this group of organisms.

SUMMARY

The *in vitro* bacteriostatic and bactericidal properties of three 9-aminoacridine derivatives, 3-chloro-7-methoxy-9-aminoacridine hydrochloride and 3-chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate (an Atabrine analogue) are presented. These compounds, whose activities are to a great extent bactericidal, exhibit their effects in high dilutions, particularly against anaerobic organisms.

The authors wish to express their appreciation to Dr. E. A. Steck for the preparation of the compounds studied.

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LABORATORY METHODS

GENERAL

FACTORS INFLUENCING THE ROMANOVSKY STAINING OF BLOOD FILMS AND THE ROLE OF METHYLENE VIOLET

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THE capriciousness of Wright's stain and its variations in staining from one sample to another have long been a matter of concern to hematologists. Many variations in technique of using the stain have been suggested, but it has still been necessary to determine the behavior of each new sample before consistent results could be obtained. Some samples do better with relatively longer "fixation" time before dilution, some with shorter staining time, and some with longer staining time after dilution. Some tolerate greater dilutions and give superior performance with them; others are ineffective under these conditions.

There are, furthermore, considerable variations in the color values attained. Samples resembling Jenner's stain give from light to fairly deep blue nuclei, blue lymphocyte cytoplasm, and good neutrophile and eosinophile granule stains but fail to stain the chromatin of blood protozoa. Then there are stains giving from deep violet to reddish purple chromatin, blue lymphocyte cytoplasm, perhaps less brilliant eosinophile granules, and good neutrophiles. A third group is recognized which gives deep red-purple chromatin, from gray to lilac lymphocyte cytoplasm, and less well-stained eosinophile granules. Generally speaking, stains of the second class are preferable both for blood morphology and for parasite identification; the third class will serve and the first group is inferior.

The color of the red corpuscles can vary from gray-green to bright orange-pink, but it is now generally recognized that any desired shade may be attained by adjustment of the pH of the diluting water. For instance, pH 7.0 gives gray or faintly orange-pink corpuscles, pH 6.8 more pink, 6.2 to 6.0 bright pink with decrease in intensity of nuclear staining; 6.5 or 6.4 is preferred by many.

The color variations in nuclear and cytoplasmic staining are fairly clearly associated with the composition of the basic component of the Romanovsky stain. Azure A tends to give deep red-purple chromatin and gray cytoplasm.[1, 2] Samples in which azure A predominates give absorption spectra with maxima

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Received for publication, Aug. 29, 1944.

around 620 to 630 mμ. Azure B gives blue cytoplasm, a more violet-purple chromatin stain, and an absorption maximum around 645 mμ. An increase in the proportion of methylene blue alters the color of chromatin staining progressively toward deep blue, and as the amount of azure further decreases, the characteristic light blue nuclear staining of Jenner's stain is reached; the absorption maximum then approaches 665 mμ.

The problem of qualitative composition of a Romanovsky stain is primarily one for the manufacturer rather than for the hematologic technician, since few users now manufacture their own stain. In the past, very wide variations have occurred in successive samples from the same manufacturer. Successive batches from manufacturer C gave absorption band medians at 658, 654, and 621, 655 and 657, 654 and 656, 657, 657, and 659 mμ; from manufacturer A, at 618, 655, and 654; from manufacturer B, at 624 and 621; from manufacturer L, at 658 and 660, 660, 659, 661, 656, 654, 655, 652, and 657, 655 and 657, 655, 656, and 658 and 659; and from manufacturer N, at 631 and 633, 640 and 655, 638 and 650, 634 and 643, 653 and 628, 652 and 656, 634 and 638, 633 and 657, 646 and 652, 654 and 657, and 652. Attempts to make samples in the laboratory by Wright's method gave even wider variation in results: 618, 618, 651, and 629 mμ. Spectrographic control aids in attaining greater uniformity, and greater uniformity has been noted in the more recent commercial samples since the suggestion that it be used was published in 1943.[3]

The selection of the proper range of composition for a Romanovsky stain for clinical laboratory use is not entirely a matter of personal preference in color values, as might be assumed from the above. It has been shown[4, 5] that the stock solutions of the eosinates of methylene blue and the azures in methanol, methanol + glycerol, and other solvents undergo progressive alteration in the direction of formation of lower azures and methylene violets. This change is faster in some solvents than others, perhaps as fast in methanol as in any, slowest in equal volume mixtures of glycerol-methanol and glycerol-ethanol. It is accelerated markedly by small amounts of alkali in methanol, retarded by acid. The presence of acid, however, disturbs the eosin-thiazin balance, so that on dilution with water the eosin is thrown out as color acid. It further appeared that thiazin eosinates composed chiefly of methylene blue and azure B eosinates remained usable far longer after solution in methanol than those in which azure A was the dominant thiazin component.

Since continued experience showed that thiazin eosinates made by the Wright[6] process continued to give results which varied from lot to lot, it was determined to explore the possibility of substituting MacNeal's[7, 8] acid chromate process as modified by Holmes and French[9] and by me[10, 11] for making azure B eosinates for the traditional sodium bicarbonate process. This latter is, properly speaking, a sodium carbonate process, since the reaction is carried out in Wright's method at about 95 to 98° C., well above the 80° C. point, at which $NaHCO_3$ breaks down to Na_2CO_3 and CO_2.

Since it had been found previously[10] that medicinal methylene blue of 85 to 90 per cent dye content required about 250 mg. of $K_2Cr_2O_7$ per gram to convert it to a crude azure B (absorption band median about 647) and that 100 mg. gave an absorption band median about 658, it was determined to make a series of polychrome methylene blue samples by boiling 5 Gm. lots of methy-

lene blue twenty minutes in 300 c.c. of approximately 0.2 M* sulphuric acid with 150 mg. potassium bichromate per gram dye. These were then cooled to 10° C. and neutralized by the cautious addition of sodium bicarbonate in precalculated amount (10.5 Gm., a little in excess of the 10.08 Gm. theoretically required). To each portion was then added 80 c.c. of 5 per cent eosin Y (NE-19) with constant shaking. After some hours the precipitates were removed by filtration through a Buchner funnel, rinsed with two 50 c.c. portions of distilled water and one 25 c.c. portion of 95 per cent alcohol. Precipitates were then dried on a warm plate at about 35° C. Eight lots of methylene blue from three manufacturers were thus used: NA-15, NA-19, NA-24, LA-7, LA-11, CA-24, and CB No. 430835 and No. 480843.

As the first of the eight was precipitated in two fractions in determining just how much eosin to use, there resulted nine lots of eosinate. As estimated from the absorption spectra in accordance with previously published tables,[12] these ranged from 21 to 28 per cent azure B to from 79 to 72 per cent methylene blue as eosinate. The medians of the absorption bands[2] were 656 and 659 mμ for the divided lot: 658, 658, 658, 658, 660, 569, and 659, respectively, for the nine lots.

These were severally dissolved in methanol at 150 mg. per 100 c.c. After three days with periodic shaking these were tested by staining methanol-fixed rat blood films containing numerous *Trypanosoma equiperdum*. Mixtures of stain and of water buffered to pH 6.5 in proportion of 1:2 (measured in cubic centimeters with graduated serologic pipettes, not by drops) were made and films were stained 3 and 5 minutes on the slide. Blue-violet to violet-purple† nuclei and blue lymphocyte cytoplasm were obtained with all, and all nine stains were deemed satisfactory. Samples of the dry stain powders were submitted also to two other observers. One of these used Wright's[13] earlier directions of 300 mg. dry stain per 100 c.c. methanol and obtained quite satisfactory results with all samples tried. The other used Conn's[14] directions of 100 mg. in 60 c.c. methanol, and a drop-for-drop dilution with water at pH 6.5, and obtained quite variable results. Two were considered very good; 3, good; 2, fair; and 2, poor. However, a second series of six samples from a single lot of polychrome methylene blue eosinate submitted to this same observer under code gave equally discordant results: 2, very good; 1, good; 2, fair; and 1, poor. Hence the variability of this observer's results would seem to be due to factors other than the dye itself. Discussion of possible factors involved will be reserved for a later point.

To determine whether a less blue tone for nuclear chromatin would be desirable, a second series of polychrome methylene blues were made by the same method, except that the potassium bichromate was increased to 200 mg. per gram methylene blue, and eosinates were prepared as before. Seven lots of medicinal methylene blue and one of the double zinc chloride were used: NA-24, M No. 41930, M No. 32718, NA-14, NA-8, NA-12, CA-21, and NA No. 9781 ($ZnCl_2$). Medians of the thiazin absorption bands fell at 653, 651, 651, 651, 651, 649, 650 and 651. Methanol solutions were made at 300 mg. per 100 c.c.

*1.16 per cent by volume of concentrated acid, specific gravity 1.84, 95.5 per cent.

†The word purple is used in this paper to designate a color between red and spectral violet.

by shaking at intervals for three days. Three-minute stains on the slide with mixtures of 1 part (0.8 c.c.) stain and 2 parts (1.6 c.c.) water buffered to pH 6.5 gave uniformly excellent staining of leucocytes and trypanosomes. Dilutions of 1 part (4 c.c.) stain to 9 parts (36 c.c.) buffered water in a coplin jar gave similarly uniformly good stains in 20 minutes.

Another series of eosinates was made by the same technique from five samples of methylene blue furnished by the National Aniline Division, four of the $ZnCl_2$ double salt (methylene blue BB) and one medicinal, using again 200 mg. $K_2Cr_2O_7$ per gram. The methylene blue BB samples yielded eosinates with absorption band medians at 649, 647, 648, and 646, approximately in the same range as medicinal methylene blue samples heated with 250 mg. $K_2Cr_2O_7$ per gram.[10] The medicinal methylene blue gave a median at 650.5, agreeing with the previous set.

Stock solutions of all were made in equal volumes of glycerol and methanol at 600 mg. per 100 c.c., and rat trypanosomiasis films were first fixed in methanol and then stained for varying periods in a 1:25 dilution in water buffered to pH 6.5. With all five samples, leucocytes were overstained and excellent details of trypanosome morphology obtained with an hour's staining; 30 minutes gave satisfactory, though less intense, staining of trypanosomes and excellent blood cell staining in all. Blood cells were quite well stained in from 10 to 15 minutes. These samples, made with no special precautions from the commercial methylene blue BB ($2C_{16}H_{18}N_3SCl \cdot ZnCl_2 \cdot H_2O$), were in no way inferior to those made from medicinal methylene blue ($C_{16}H_{18}N_3SCl$), the only difference being due to the lower dye content consequent on the presence of 1 molecule of $ZnCl_2$ (MW:136.3) and 1 molecule of water (MW:18) for each 2 molecules of methylene blue (MW:319.7), a proportion of 80.5 per cent methylene blue. The chemical data are derived from the *Colour Index*.[15]

It was previously observed on several occasions that Romanovsky staining was unsatisfactory in coplin jars when the same proportions of stock methanol solution and buffered water were used as give satisfactory stains on the slide. Hence, it was decided to explore further the limitations imposed by the final concentration of methanol in the diluted stain.

Two lots of ploychrome methylene blue eosinate, both made by the foregoing acid chromate process, were used in stock methanol solution, 44 D at 300 mg. per 100 c.c.; the other, 44 B, at 150 mg. per 100 c.c. Rat trypanosomiasis blood films were previously fixed from 2 to 3 minutes in methanol and stained in coplin jars for varying intervals. Into each of seven jars were placed 4 c.c. of stock stain. In the first, 36 c.c. water were added; in the second, 1 c.c. methanol and 35 c.c. water; in the rest, 2, 3, 4, 5, and 6 c.c. methanol with water to make 40 c.c. total as before. This gave final methanol percentages ranging by 2.5 per cent steps from 10 per cent to 25 per cent. All jars were covered during staining. With both samples placed in the first and second jars (at 10 and 12.5 per cent methanol), excellent and good stainings were attained respectively in 1 and 2 hours, quite good and fair in 30 minutes. The same grade of staining with each sample was attained in 2 hours at 15 per cent methanol, but staining in 1 hour was inferior. Fair staining only could be obtained in 2 hours at 17.5 per cent methanol, and in from 20 to 25 per cent methanol, nuclei remained pale blue even with 2 hours' staining.

It would appear that the upper limit for methanol concentration in coplin jar staining was between 12.5 and 15 per cent.

In the last series the stock solution containing 300 mg. per 100 c.c. gave consistently better results than that containing only 150 mg. per 100 c.c. This indicates that the final concentration of 0.15 mg. per cubic centimeter is too low to be effective in from 1 to 2 hours' staining, while 0.3 mg. per cubic centimeter gives good results in 30 minutes and excellent in 1 hour.

Since the concentrations of methanol in staining blood films in the usual manner are initially far higher than those which permit staining in a covered jar, a series of tests was made to see whether prevention of evaporation or its acceleration would influence staining on the slide. Dilutions were 1 part (0.8 c.c.) of stain and 2 parts (1.6 c.c.) of water and 1 (0.6 c.c.) of stain and 3 (1.8 c.c.) of water, buffered as usual at pH 6.5. Stains done in the open with or without an electric fan blowing were satisfactory, but when staining was done in the same manner under a bell jar, where the atmosphere was previously saturated with methanol vapor, cell nuclei remained pale blue.

Another series was done at a 1 part stain to 3 parts water dilution of another acid chromate (200 mg. per gram), polychrome methylene blue eosinate (44F). Rat trypanosomiasis blood films were first fixed in methanol for from 2 to 3 minutes immediately after making. Then they were stained 2, 3, 5, and 8 minutes. In one series, 0.5 c.c. stain (300 mg. per 100 c.c.) was deposited on the slide, and 1 minute later, 1.5 c.c. water buffered to pH 6.5 were added, while in another series stain and buffered water were first mixed in a test tube and then deposited on the slide. This second series generally showed better and more uniform staining than the first.

In some of those preparations mixed on the slide, mixing was accomplished by rocking the slide, in some by a gentle air stream, both moist and dry air being tried. Little difference was apparent among these. Some films were stained in quiet air with doors and windows closed; others, with an electric fan blowing directly across the staining rack. This again made little evident difference. However, placing the films on a warm plate at 37° C. during staining accelerated it somewhat and definitely increased the intensity. One repetition of the methanol moist chamber experiment gave only pale blue nuclei; another gave fairly good results. This chamber was not designed to maintain saturation particularly well, so the one successful stain of the three trials is probably significant rather of a lowered methanol vapor content of the air in the chamber than of a negation of the other two experiments.

Since sharper nuclear staining and more vivid color contrasts are obtainable by Giemsa type stains than by the usual Wright stain, it was determined to try this lot 44F also in glycerol-methanol stock solution at 1:24 and 1:49 dilutions. To estimate the proper dye content for such a stock solution, 50 c.c. quantities of an equal volume mixture of methanol and glycerol were placed in each of twelve bottles and to these were added, respectively, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1 Gm. of polychrome methylene blue eosinate 44F. When dilution (d) × optical density (D) at both the absorption maxima, thiazin and eosin, were plotted against milligrams per cubic centimeter of dry dye placed in the solvent, graphs were obtained which formed straight lines from 0 to 8 mg. per cubic centimeter. At 8 mg. per cubic centi-

meter, d × D = 580 for the thiazin, 520 for the eosin. Beyond those points, d × D values were irregular, and less than predicted from the graph of lower values, reaching maxima of 764 and 705, respectively. Assuming that these maxima represent saturation values, the straight line graphs intersect the horizontals for these saturation values at 10.9– and 10.9+ mg. per cubic centimeter, respectively, for the thiazin and eosin graphs, a very close agreement. It would seem to be indicated from these figures that at the temperatures of 25+° C. then prevailing, equal volumes of glycerol and methanol will dissolve nearly 11 mg. of this polychrome methylene blue eosinate per cubic centimeter of solvent.

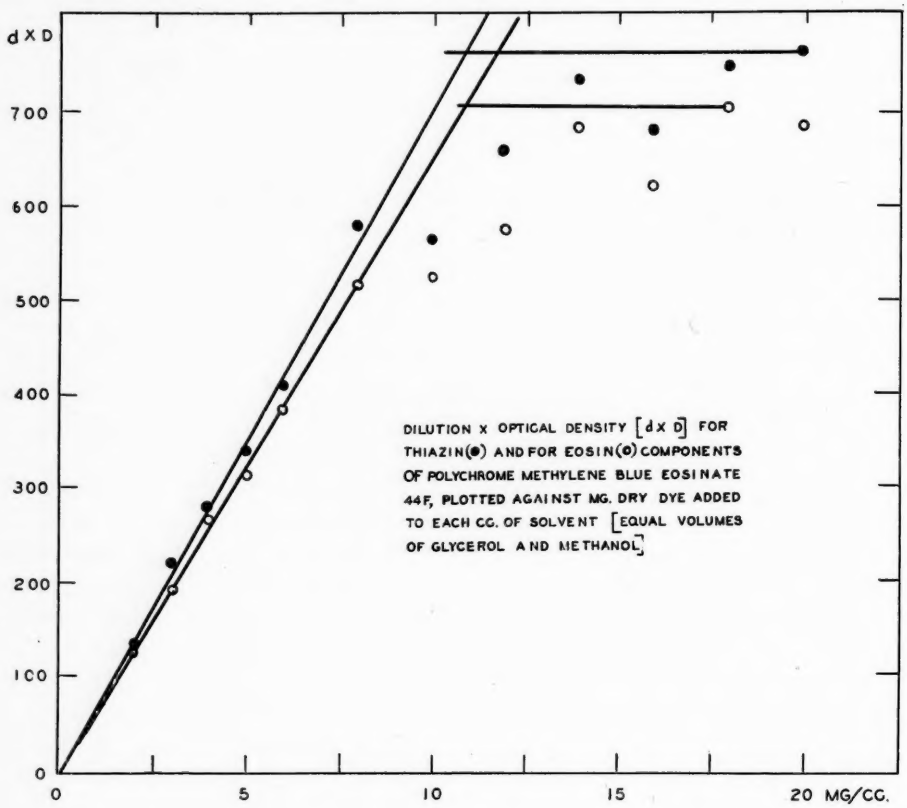

Fig. 1.

All of these stains were tested by staining rat trypanosomiasis blood films in coplin jars at 1:49 dilution at pH 6.5 for varying periods. The solutions in which 0.8 per cent dye or more was used all gave excellent results for both leucocytes and trypanosomes in 30 minutes. The three with thiazin dilution × density (d × D) values above 700 gave excellent results in 10 minutes; the two with d × D values between 600 and 700 required 15 minutes; those with d × D between 500 and 600 required from 20 to 30 minutes.

With concentrations from 0.8 per cent down, the d × D values corresponded to the amount of dye added. The 0.6 per cent solution required 30 minutes at 1:24 dilution or 1 hour at 1:49 for excellent results; the 0.5 per

cent the same; the 0.4 per cent, 4 hours at 1:49 or 30 minutes at 1:24; the 0.3 per cent was ineffective at 1:49, but stained well at 1:24 in from 1 to 2 hours; and the 0.2 per cent was inferior in 2 hours at 1:24 but gave excellent results at 1:9 in 20 minutes.

It has been observed that 1:24 and 1:49 dilutions of from 0.8 to 1.0 per cent glycerol-methanol solutions of eosinates of methylene blue polychromed by the acid chromate process are fairly stable and often can be used for a second batch of slides after a first has been stained. Accordingly, successive lots of six slides were stained at 30-minute intervals in a single 50 c.c. lot of a 1:24 dilution of 1 per cent glycerol-methanol solution of polychrome methylene blue eosinate 44F. Of these, two slides were stained 10 minutes, two for 15 minutes, and two for 20 minutes. In all, seventy-eight slides were thus stained over a period of 7½ hours. Of these, the best were the first two stained 20 minutes, but quite acceptable stains were obtained with 20-minute staining up to the fifth group. Longer staining probably would have given better results, but the 30-minute interval for starting a fresh lot did not permit this. This gradual loss of staining power is due both to exhaustion of stain and to spontaneous weakening of the solution by precipitation. A similar batch of stain used for staining after 7 hours' standing without being used gave better results than that in which seventy-two slides had been stained before, but the results were definitely inferior even to the seventh set stained in the 3-hour-old solution after thirty-six previous slides.

Following these experiences, attempts were made to apply the method of staining with a 1:24 dilution of a 1 per cent glycerol-methanol stock solution to commercial samples of Wright's stain. Stock solutions were thus prepared of lots AWr-2 and AWr-3. One set of rat trypanosomiasis blood films were stained 5, 7.5, 10, 15, 20, and 30 minutes in 1:24 dilutions, then another in 1:9 dilutions. Pale blue nuclei, failure to stain parasite chromatin, and slight and much precipitation, respectively, resulted in both trials. Parts of the 1 per cent stock solutions were then diluted with 4 volumes of methanol to 0.2 per cent, and other films were then stained on the slide in the usual way with 0.5 c.c. dilute stock stain to 1.5 c.c. buffered water. Good results were obtained.

In search of a possible explanation for this phenomenon, 10 c.c. of the glycerol-methanol stock solution was diluted with 88 c.c. distilled water and 2 c.c. glacial acetic acid and shaken with successive portions of carbon tetrachloride until no more red color was extracted. The CCl_4 was then shaken with two or three changes of saturated sodium carbonate solution, extracting from it a deep orange-red dye with yellow fluorescence on dilution and a spectroscopic absorption maximum in water at 516 mμ, which agrees perfectly with eosin Y. The still red CCl_4 was then evaporated off on a water bath and the residue taken up in alcohol, to which it gave a deep violet color. This gave a spectral absorption peak at about 595 in alcohol. The acetic aqueous residue was blue violet in color and gave a single absorption maximum at 660 mμ (median of absorption band at 658).

The three fractions were regarded respectively as (1) eosin, (2) methylene violet, and (3) azures and methylene blue. It has been known since Bernthsen's time[16] that on chloroform extraction of alkaline solutions of polychrome methy-

lene blue, azures and methylene violet pass into the chloroform, and when this in turn is shaken with dilute aqueous acetic acid, the azures pass into the weak acid while the methylene violet remains in the chloroform. Further, it is stated[17] that with ether or chloroform extraction of an acetic acid solution, the azures remain in the aqueous solution, while the methylene violet passes into the chloroform or ether. The sodium carbonate wash was introduced purely to remove acetic acid from the fat solvent, and the appearance of a red dye in it was unexpected. On direct trial it was found that ether extraction of a 1 per cent acetic acid solution of eosin extracted a yellow coloring matter and that this ether solution imparted an orange-red color to aqueous sodium carbonate solution.

Recombination of the thus prepared azure methylene blue fraction with the eosin fraction, from the above Wright's stain, omitting the methylene violet fraction, gave satisfactory Romanovsky staining of blood films in a coplin jar when the dilution was of about the same depth of color as the 1:24 dilutions of the 1 per cent glycerol-methanol stock solution.

Since the foregoing indicated that methylene violet might be the substance responsible for the failure of certain samples to stain in coplin jar staining, it was determined to test this hypothesis further. To this end further trials of the fractionation method used above were made and chloroform substituted for ether or carbon tetrachloride, since it appeared to extract methylene violet much more rapidly. After a number of trials, the following method was evolved:

Dissolve 500 mg. Wright's stain in 25 c.c. methanol and 25 c.c. glycerol by shaking at frequent intervals for three days. Let settle over night. Pipette out 10 c.c. into a 120 c.c. bottle; add 88 c.c. distilled water and 2 c.c. glacial acetic acid. Mix thoroughly and let stand over night. Filter and shake filtrate in a separatory funnel with successive 50 c.c. portions of chloroform until last portion is pale pink or bluish. Combine chloroform extracts and shake with two successive 20 c.c. portions of 2 M sodium carbonate in a large separatory funnel. Combine the sodium carbonate fractions and neutralize cautiously with about 9 c.c. glacial acetic acid until further addition causes no further evolution of gas. Label and store this as "Eosin fraction of Wright's stain lot No. ---." Distill and recover the chloroform, using a water bath to heat the distilling flask. Take up the residue in successive 10 c.c. portions of absolute ethyl alcohol, filtering through filter paper to remove sodium salts, and make up final volume to 50 c.c. Label and store this as "Methylene violet fraction of Wright's stain lot No. ---." Store the aqueous acetic solution from which the chloroform extract was made in a 120 c.c. bottle, labeling "Azure fraction of Wright's stain, lot No. ---." Record total volume in cubic centimeters of each fraction, also dilution for spectrophotometry and spectroscopic data: λ (the absorption maximum), width of absorption band in which density is over 90 per cent of its maximum, the median of this band, and the optical density D at λ.

Since it appeared that the only adequate sample of commercial methylene violet on hand at the time contained a considerable proportion of azures, 5 Gm. of this was dissolved in some 1,200 to 1,500 c.c. of 2 per cent acetic acid and

extracted with chloroform, the chloroform extract was dried by distillation over a water bath, the acetic acid removed with a small amount of aqueous sodium carbonate solution, the again dry residue taken up in absolute alcohol, filtered, evaporated to dryness, removed as completely as possible, weighed to the nearest 0.1 mg., and dissolved in equal volumes of glycerol and methanol at 0.5 per cent concentration. The total yield was about 450 mg. This stock solution was diluted in appropriate amount and dilution for spectroscopic examination and the optical density determined and related to the amount of dry dye for comparison with the methylene violet fractions from the Wright's stain samples. This enabled the calculation in milligrams of the amount of methylene violet fraction recovered from the 10 c.c. of stock glycerol-methanol solution, which contained 100 mg. of Wright's stain.

Next, rat trypanosomiasis blood films were stained in a 1:24 dilution of each 1 per cent glycerol-methanol stock solution for 5, 7, 10, 15, 20, 30, 60, and 120 minutes in coplin jars, and on the slide in 2 parts of buffered water and 1 part of a dilute stock stain consisting of 2 c.c. of the 1 per cent glycerol-methanol solution and 8 c.c. methanol for 1, 2, 3, 5, 7.5, and 10 minutes. Water buffered to pH 6.5 was used throughout. All slides were examined under oil immersion and a numerical grade given in accordance with the following scheme:

7 Very dark cell nuclei, dark purple solid trophonuclei, heavy pink undulating membrane
6 Rather dark cell nuclei, solid trophonuclei, pink undulating membrane
5 Violet-purple cell nuclei, granular purple trophonuclei, clearly perceptible undulating membrane
4 Violet-purple cell nuclei, partially stained trophonuclei, well-stained blepharoplasts, indistinct undulating membranes
3 Rather light purple or red-purple nuclei, suggestion of trophonuclei, distinct blepharoplast
2 Blue-violet (–) → violet → violet-purple (+) cell nuclei, trophonuclei not shown, blepharoplasts, variable – to +
1 Blue nuclei deeper than cytoplasm, trophonuclei and blepharoplasts not shown
0 Unstained to pale blue or gray nuclei

Grade 7 is distinctly overstained; grades 5 and 6, good for trypanosomes; grades 4 and 5, good for blood work; grade 3, usable, grade 2, inferior; grades 1 and 0, useless.

The performance of two laboratory and twenty-six commercial samples of Wright's stain from four manufacturers, designated as A, B, D, and E, is given in Table I along with the assay figures for methylene violet recovered from a 100 mg. sample by the method given previously. Samples giving less than 4 mg. methylene violet gave satisfactory staining in coplin jars at 1:24 dilution of the 1 per cent glycerol-methanol solution. Some samples yielding around 4 mg. gave good staining after longer than the usual interval, while others did not. Samples with 5 mg. or more failed to give satisfactory stains in coplin jars. All but one sample gave satisfactory stains on the slide with a 1:2 dilution of an 0.2 per cent solution in 90 per cent methanol-10 per cent

glycerol (made by diluting the 1 per cent glycerol-methanol stock solution with 4 volumes of methanol).

Following these experiences it was decided to determine the effect of adding varying amounts of the purified methylene violet described above to the crude azure eosinate 44F. This last was made by acid chromate oxidation and should, according to Holmes,[17] contain no methylene violet. The assay (Table I) indicated that only traces were present. In a previous similar trial a commercial methylene violet was used. It did not interfere with azure eosinate staining in jars and gave the gray-lilac cytoplasm and poor nuclear staining of azure C eosinate when higher proportions were used. A relatively large proportion of it remained in dilute acid aqueous solution when extracted by shaking with ether or chloroform. Consequently a methylene violet made by extracting with chloroform from a dilute acetic acid solution was used.

A series of stains was done in coplin jars with 2 c.c. 1 per cent glycerol-methanol solution of azure eosinate 44F in 48 c.c. of water buffered to pH 6.5, with the addition of graded amounts of an 0.5 per cent solution of repurified methylene violet in glycerol and methanol (equal volume mixture). The control series gave excellent stains, grade 6, on the grading schedule given above

TABLE I

WRIGHT'S STAIN PERFORMANCE IN COPLIN JAR AND "ON SLIDE" STAINING, CORRELATED WITH CRUDE METHYLENE VIOLET ASSAY

DYE	METHYLENE VIOLET MG. RECOVERED FROM 100 MG. DYE	COPLIN JAR 1:24 DILUTION OF 1% GLYCEROL-METHANOL STOCK		ON SLIDE 1:2 DILUTION OF 0.2% IN 90% METHANOL-10% GLYCEROL		ON SLIDE STAINING NOTES AND OTHER DILUTIONS
		BEST GRADE	TIME MINUTES	BEST GRADE	TIME MINUTES	
A1		1	5 to 90	4 to 6	2 to 12	1:3 instead of 1:2
A2	5.71	1	5 to 90	5 to 6	2 to 12	1:3 instead of 1:2
B1	4.83	1	5 to 120	5	1 to 10	1:4 to 1:2 in 1 to 10 min.
B2	6.15	1	5 to 120	5	1 to 10	1:4 to 1 in 1 to 10 min.
B3	4.29	1	5 to 120	4 to 6	1 to 10	1:4 to 1:1 in 1 to 10 min.
B4	5.08	1	5 to 120	4 to 6	2 to 10	1:4 to 1 in 1 to 10 min.
B5		1	5 to 30	1	2 to 12	1:3 instead of 1:2
B6	0.60	5	15 to 30	4 to 6	2 to 12	1:3 instead of 1:2
C1	1.53	4 to 6	30 to 120	4 to 5	2 to 10	
D1	1.57	4 to 5	15 to 30	4 to 6	3 to 12	1:3 instead of 1:2
D2	1.51	5 to 6	7.5 to 120	4 to 5	2 to 10	
D3	2.64	4 to 5	7.5 to 120	5	1 to 10	
D4	1.99	5 to 6	7.5 to 120	4 to 5	1 to 10	
D5	1.01	4 to 5	15 to 30	4 to 6	2 to 12	1:3 instead of 1:2
D6	1.30	5 to 7	5 to 120	4 to 5	2 to 10	
D7	1.54	5 to 6	7.5 to 120	4 to 5	2 to 10	
D8	1.66	5 to 6	5 to 120	4 to 5	1 to 10	
E1	4.81	4	60 to 120	1 to 4	1 to 10	1:4 to 3:4 in 5 to 10 min.
E2	3.88	4 to 5	60 to 120	5 to 6	1 to 10	1:4 to 5 in 2 to 10 min.
E3	4.01	4 to 5	60 to 120	4 to 5	1 to 10	1:4 to 5 in 2 to 10 min.
E4	4.97	1	5 to 120	4 to 6	1 to 10	1:4 to 4:5 in 1 to 10 min.
E5	3.95	1 to 4	120	4 to 6	1 to 10	1:4 to 5 in 3 to 10 min.
E6	4.31	1	5 to 120	4 to 6	2 to 10	1:4 to 5 in 2 to 10 min.
E7	6.35	1	5 to 120	5	1 to 10	1:4 to 4 in 10 min.
E8	5.91	1	5 to 30	5 to 6	2 to 12	1:3 instead of 1:2
E9	4.31	1	5 to 120	4 to 5	1 to 10	1:4 to 5 in 3 to 10 min.
E10	3.85	1	5 to 120	4 to 5	2 to 10	1:4 to 3:6 in 1 to 10 min.
44F	0.06	4 to 6	7.5 to 30	5	2 to 12	1:3 instead of 1:2

in all periods from 15 minutes up to 2 hours. The series receiving 0.2 c.c. (1 mg.) (about 5 per cent of total dry stain) methylene violet gave a good stain, grade 5, in 2 hours, inferior stains in lesser intervals (grade 4 in 60 and 30 minutes, grade 2 in 20, and grade 1 in shorter intervals). With larger amounts of methylene violet (2, 3, 4, 5, 6, and 7 mg.) poor stains were regularly obtained.

This test was repeated, using 0.1, 0.15, 0.2, 0.25, 0.3, and 0.4 c.c. of the 0.5 per cent glycerol-methanol solution of methylene violet with 2 c.c. 1 per cent azure eosinate 44F and 48 c.c. water buffered to pH 6.5. In the control series, grade 5 or grade 6 stains were obtained in all periods from 15 to 120 minutes. With 0.1 c.c. (= 0.5 mg. or 2.5 per cent) methylene violet, similarly satisfactory stains were obtained in 30, 60, and 120 minutes but not in shorter times. With 0.15 c.c., the satisfactory stains, grade 5, were obtained only after 60 and 120 minutes. With 0.2 c.c. (= 1 mg.) a single satisfactory stain was obtained in 1 hour; those stained 30 and 120 minutes gave irregular, chiefly poor, results. With larger amounts of methylene violet no satisfactory stains were obtained.

A third similar test was made using azure eosinate 44F and methylene violet in such proportion that a total of 100 mg. dry dye per 100 c.c. glycerol-methanol stock solution was present. The first contained 100 mg. azure eosinate 44F alone; in the second this was reduced to 97.5 mg. and 2.5 mg. methylene violet added; and further mixtures contained 5, 7.5, and 10 mg. methylene violet with corresponding reduction of the azure eosinate. Of these stock mixtures there were made (A) a 1:24 dilution in water buffered to pH 6.5; (B) a 1:24 dilution containing 8 per cent acetone in the buffered water; and (C) a 1:49 dilution with 8 per cent acetone in buffered water.

The three controls without methylene violet gave (grades 5 and 6) excellent stains in from 10 to 120 minutes. With 2.5 per cent methylene violet, the acetone mixtures gave excellent stains in from 10 to 120 minutes; that without acetone, in from 15 to 120 minutes. With 5 per cent methylene violet all three mixtures stained moderately well in from 30 to 120 minutes, and with 7.5 per cent and 10 per cent methylene violet satisfactory stains were not obtained up to 120 minutes with any of the mixtures.

Since the foregoing trials indicated that this methylene violet seriously interfered with staining in coplin jars, it was decided to discover whether it had any deleterious effect in "on slide" staining in the traditional manner. Accordingly, a series of dilutions of the foregoing stock mixtures in methanol was made, bringing the dye concentration down from 1 per cent to 0.2 per cent (200 mg. per 100 c.c.). Three series were stained for 1, 2, 3, 5, 7.5, and 10 minutes. The first was diluted 4 c.c. stain to 8 c.c. water; the second, 3 c.c. stain to 9 c.c. water; the third, 2.5 c.c. stain to 10 c.c. water. All the water was buffered to pH 6.5. Each series comprised mixtures of azure eosinate with 0, 2.5, 5, 7.5, 10, 12.5, 15, 20, and 25 per cent methylene violet.

The first dilution series, 1 stain plus 2 water, gave good stains in all periods from 2 to 10 minutes, regardless of the amount of methylene violet. The second series, 1 stain plus 3 water, gave good stains in from 2 to 10 minutes with methylene violet levels up to 12.5 per cent, varying to poor with

higher levels. In the third series, 1 stain plus 4 water, good results were obtained in the controls and with 2.5, 5, and 7.5 per cent methylene violet, variable with 10, 12.5, and 15 per cent, and poor with 20 and 25 per cent. None of the stains were fully satisfactory with 1-minute staining, but with 2 minutes or more, longer periods gave little increase in density of staining. All films were fixed in methanol prior to staining, and all stain-water mixtures were made in test tubes and at once pipetted onto the slides. Uniform mixtures were thereby assured.

As a further control, this last experiment was repeated, using a freshly obtained commercial sample of methylene violet (NLv-4) at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 25, and 30 per cent levels. With 1:24 dilution of the glycerol-methanol stock solution, no levels above 10 per cent were tested, since fully satisfactory staining was obtained only with 4 per cent or less methylene violet. Good staining in prolonged intervals was obtained in from 5 to 7 per cent, and in 8 and 10 per cent satisfactory staining was not attained in 4 hours.

With the stock solutions diluted to 0.2 per cent with methanol, dilutions of 1:4, 1:3, 1:2, and 5:7 were employed and the 5 and 7 per cent methylene violet levels omitted. All slides were studied at the 0, 3, 6, 10, 15, and 30 per cent levels; in addition, all the 1:4 and 5:7 dilution slides were also studied at the 20 and 25 per cent levels and the 1:4 dilution slides at the 12 per cent level. Three- and 5-minute stains were made of all the 1:2 dilution slides.

The 1:4 dilution gave good stains up to 10 per cent methylene violet, fair and variable at from 12 to 25 per cent, and poor at 30 per cent. The 1:3 and 1:2 dilutions gave good stains at all methylene violet levels, and with the 5:7 dilution, higher methylene violet levels actually gave somewhat better staining than lower concentrations, particularly with shorter staining intervals. The same difference was seen to a less degree with the 1:2 dilution. With the unmodified (control) azure eosinate, 1:4, 1:3, and 1:2 dilutions required 3 minutes for satisfactory stains, while 5 minutes were needed with the 5:7 dilution. With higher methylene violet concentration, 3- and sometimes 2-minute stains were satisfactory with the 1:3, 1:2, and 5:7 dilutions.

The dilutions used in the two last cited experiments lie within the range of those attained by the drop-for-drop dilution technique commonly used. Wright[6] originally directed placing some of the methanol solution on the preparation to fix it for 1 minute, and then added distilled water until a metallic scum formed. Later[18] he directed adding a noted quantity of stain from a medicine dropper and a minute later the same quantity of water also from a medicine dropper; in 1924[19] he directed the use of 10 drops of the stain and 10 drops of water. Mallory[20] later returned to the use of equal quantities from a pipette, while Conn[14] copied the 1924 directions of Wright. Many workers, however, do not realize that an equal number of drops is not an equal quantity when a methanol solution is compared with water.

The number of drops per cubic centimeter and average drop size in cubic millimeters for a 0.3 per cent methanol solution of azure eosinate, for a 1.0 per cent glycerol-methanol solution of the same azure eosinate, and for distilled water is listed in Table II. Ten serologic pipettes were used, five with blunt and five with sharp points; five bulb pipettes were used in two positions, nearly

TABLE II

NUMBER OF DROPS PER CUBIC CENTIMETER AND DROP SIZES FOR VARIOUS DROPPERS
SOLUTIONS OF AZURE EOSINATES

TYPE	NO.	0.3% METHANOL			1.0% GLYCEROL METHANOL			DISTILLED WATER		
		TOTAL DROPS AND C.C. COUNTED	NUMBER DROPS PER 1 C.C.	C.MM. 1 DROP	TOTAL DROPS AND C.C. COUNTED	NUMBER DROPS PER 1 C.C.	C.MM. 1 DROP	TOTAL DROPS AND C.C. COUNTED	NUMBER DROPS PER 1 C.C.	C.MM. 1 DROP
Blunt	1	175 = 4 c.c.	43.7	22.9	158 = 4 c.c.	39.5	25.3	48 = 3 c.c.	16.0	62.5
serologic	2	181 = 4 c.c.	45.2	22.1	156 = 4 c.c.	39.0	25.6	48 = 3 c.c.	16.0	62.5
1 c.c.	3	179 = 4 c.c.	44.7	22.3	158 = 4 c.c.	39.5	25.3	48 = 3 c.c.	16.0	62.5
pipette	4	178 = 4 c.c.	44.5	22.5	157 = 4 c.c.	39.2	25.5	49 = 3 c.c.	16.3	61.3
	5	134 = 3 c.c.	44.7	22.4	155 = 4 c.c.	38.7	29.2	48 = 3 c.c.	16.0	62.5
Sharp	6	169 = 3 c.c.	56.3	17.8	1,253 = 3 c.c.	49.0	20.4	66 = 3 c.c.	22.0	45.5
serologic	7	177 = 3 c.c.	59.0	17.0	1,190 = 3 c.c.	49.3	20.3	67 = 3 c.c.	22.3	44.9
1 c.c.	8	179 = 3 c.c.	59.7	16.8	1,208 = 3 c.c.	50.7	19.7	69 = 3 c.c.	23.0	43.5
pipette	9	173 = 3 c.c.	57.7	17.3	1,065 = 3 c.c.	47.7	21.0	66 = 3 c.c.	22.0	45.5
	10	171 = 3 c.c.	57.0	17.6	1,173 = 3 c.c.	48.0	20.8	66 = 3 c.c.	22.0	45.5
Bulb	11	1,422 = 25 c.c.	56.9	17.6	1,105 = 25 c.c.	19.9	19.9	577 = 25 c.c.	23.1	43.3
pipette	12	1,284 = 25 c.c.	51.4	19.5	1,067 = 25 c.c.	47.6	21.0	528 = 25 c.c.	21.1	47.3
held	13	1,273 = 25 c.c.	50.9	19.6	957 = 25 c.c.	48.7	20.5	589 = 25 c.c.	23.5	42.4
nearly	14	1,100 = 25 c.c.	44.0	22.7	1,065 = 25 c.c.	42.6	23.5	464 = 25 c.c.	18.6	53.9
vertical	15	1,203 = 25 c.c.	48.2	20.8	1,173 = 25 c.c.	46.9	21.3	495 = 25 c.c.	19.8	50.5
Bulb	16	1,199 = 25 c.c.	48.0	20.8	1,105 = 25 c.c.	44.3	22.6	580 = 25 c.c.	23.2	43.1
pipette	17	962 = 25 c.c.	38.5	26.0	1,067 = 25 c.c.	42.7	23.4	460 = 25 c.c.	18.4	54.3
held	18	1,010 = 25 c.c.	40.4	24.7	957 = 25 c.c.	38.3	26.1	501 = 25 c.c.	20.0	49.9
nearly	19	935 = 25 c.c.	37.4	26.7	999 = 25 c.c.	40.0	25.0	405 = 25 c.c.	16.2	61.7
horizontal	20	961 = 25 c.c.	38.4	26.0	1,135 = 25 c.c.	45.4	22.6	467 = 25 c.c.	18.7	53.5
Flat-	21	1,449 = 40 c.c.	36.3	27.6	978 = 30 c.c.	32.6	30.7	414 = 30 c.c.	13.8	72.5
topped	22	1,089 = 30 c.c.	36.3	27.6	1,012 = 30 c.c.	33.7	29.6	407 = 30 c.c.	14.3	73.7
dropping	23	1,005 = 30 c.c.	33.5	29.9	973 = 30 c.c.	32.4	30.9	429 = 30 c.c.	14.3	70.0
bottles	24	1,042 = 30 c.c.	34.7	28.8	982 = 30 c.c.	32.7	30.6	423 = 30 c.c.	14.1	71.0
	25	1,045 = 30 c.c.	34.8	28.7	980 = 30 c.c.	32.7	30.6	420 = 30 c.c.	14.0	71.4
Rabbit-	26	962 = 25 c.c.	38.5	26.0	918 = 25 c.c.	36.7	27.2	381 = 25 c.c.	15.2	65.6
ear-	27	976 = 25 c.c.	39.0	25.6	898 = 25 c.c.	35.9	27.8	402 = 25 c.c.	16.1	62.2
topped	28	950 = 25 c.c.	38.0	26.3	886 = 25 c.c.	35.5	28.2	371 = 25 c.c.	14.8	67.4
dropping	29	950 = 25 c.c.	38.0	26.3	930 = 25 c.c.	37.2	26.9	341 = 25 c.c.	13.6	73.3
bottles	30	1,068 = 25 c.c.	42.7	23.4	1,017 = 25 c.c.	40.7	24.6	185 = 25 c.c.	7.4	135.0
	30	1,023 = 25 c.c.	40.9	24.4	918 = 25 c.c.	36.7	27.2	347 = 25 c.c.	13.9	72.0
	30							202 = 25 c.c.	8.1	123.7
	30							175 = 25 c.c.	7.0	142.8
	30							227 = 25 c.c.	9.1	110.2
	30							305 = 25 c.c.	12.2	82.0

vertical and nearly horizontal, and ten dropping bottles of two different designs, five each, were tried. With the last of these, No. 30, six consecutive trials were made with water, two each with the two stain solutions.

Taking the extremes in drop sizes, a drop-for-drop dilution of the methanol solution could vary from 41 per cent stain or 1:1.44 to 10.5 per cent or 1:8.5. If bottle 30, which gave extraordinarily large drops of water, were neglected in the calculation, the lower dilution would be 18.6 per cent or 1:4.39. This last dilution approaches a level where stains with higher than 7.5 per cent methylene violet do poorly in "on slide" staining.

It may be noted further that the traditional 1 drop per 1 c.c. dilution for Giemsa stain would range, on the basis of the azure eosinate solution in equal volumes of glycerol and methanol, from 1:33.4 to 1:50.8. This variation is enough to make a very considerable difference in the density of staining obtainable in from 30 to 45 minutes, particularly with reference to such structures as Schüffner's granules and undulating membranes.

Since some authors have recommended equal volumes of Wright's stain and water for staining (though I am sure they meant an equal number of drops), it was decided to determine the effect of such high concentration of methanol solution. Some years ago we tried 1:1 dilutions and abandoned them for a 1:2 dilution because of inferior performance.

A 0.3 per cent methanol solution of azure eosinate 44D was used. Mixtures with buffered water (pH 6.5) were made in test tubes containing, respectively, 60, 55, 50, 45, 40, 35, and 33 per cent by volume of the stock stain, and 1.5 c.c. quantities at once deposited on previously fixed rat trypanosomiasis blood films. These were stained 1, 2, 3, 5, 7, 5, and 10 minutes. Generally, all the 1-minute films were inferior (grade 2 on the grading schedule). The 33 per cent dilution gave good stains in periods of 2 minutes or more, with fair to good differentiation of trypanosome nuclei in periods of from 5 to 10 minutes. At 45 per cent dilution, trypanosome trophonuclei were poorly stained in from 7.5 to 10 minutes, with fairly good blood staining. With 50 to 60 per cent stain, trophonuclei were not stained, and leucocyte staining was only fair. Really satisfactory blue lymphocyte and trypanosome cytoplasm was not attained at any concentration higher than 33 per cent. With increasing concentration, cytoplasm altered in color through lilac to pinkish gray.

SUMMARY

The constitution of the azure component of Wright's stain is difficult to control when the traditional process of steaming one hour with $NaHCO_3$ is used. This difficulty is largely overcome in commercial manufacture by spectrographic control.

Substitution of the acid chromate oxidation process gives relatively constant azure constitution when the same proportions of methylene blue and potassium (or sodium) bichromate are used.

Preparation of thiazin eosinates containing chiefly azure B and methylene blue is suggested on account of the greater stability of these in methanol solution than azure A eosinates.

Satisfactory azure eosinates are readily prepared from the zinc chloride methylene blue. In calculation of molar equivalents, the presence of 19.5 per cent of water and zinc chloride must be allowed for.

There are distinctly different limitations on staining in covered jars and on open slides. In covered jars methanol percentages of 15 per cent or more are distinctly inhibitory to azure staining of nuclei, while on open slides 33 per cent methanol is tolerated. Here, too, higher than 35 per cent methanol definitely impairs staining of leucocytes and especially parasites. When evaporation of methanol is retarded in "on slide" staining, the usual 25 to 33 per cent dilutions fail to stain them.

Distinctly sharper staining of chromatin and clearer cytoplasmic colors are obtained by longer staining in more dilute solutions, even when the same thiazin eosinate is employed.

The solubility of a mixed methylene blue azure B eosinate in equal volumes of methanol and glycerol is in the neighborhood of 1.1 per cent or 11 mg. per cubic centimeter.

Quite satisfactory staining may be attained in from 15 to 20 minutes with a 1:24 dilution of a 1 per cent glycerol-methanol solution of such an eosinate in covered jar staining. Such solutions may be re-used but with somewhat inferior results.

Many samples of commercial Wright stain do not permit staining by this technique; others perform beautifully. It was found that the first group of samples contained higher proportions of methylene violet than the second. In confirmation of this finding it was shown that chemical fractionation of a Wright stain of the first group into azure, eosin, and methylene violet fractions and recombination of the first two fractions would give successful staining from dilute aqueous solution. It was further shown that addition of purified methylene violet to a 1 per cent glycerol-methanol solution of an azure eosinate in 2.5 per cent level retarded satisfactory staining in the 1:24 aqueous dilution, with 5 per cent retardation was greater, and with 7.5 per cent satisfactory staining was inhibited. With a commercial methylene violet 4 per cent was tolerated, from 5 to 7 per cent retarded staining, and from 8 to 10 per cent inhibited.

However, when a 200 mg. per 100 c.c. methanol solution of the same azure eosinate was used with 1:2, 1:3, and 1:4 dilutions in "on slide" staining, the 1:2 dilution gave satisfactory stains with substitution of as much as 25 per cent methylene violet, the 1:3 dilution tolerated methylene violet substitution up to 12.5 per cent of the total dry dye, and the 1:4 dilution up to 7.5 per cent only. Higher levels of a commercial methylene violet were tolerated and appeared to improve staining with higher initial methanol concentration in the final stain mixture.

It would appear that methylene violet is kept in solution by the presence of methanol rather than by the azures, as previously assumed, and that when insufficient methanol (or other solvent) is present to keep the methylene violet in solution, it precipitates and carries the azure eosinates down with it.

Drop-for-drop dilutions of methanol solutions of azure eosinates with water do not constitute equal volume mixtures but vary from about 1:1.5 to 1:4.5 (41 per cent to 19 per cent).

One drop to 1 c.c. dilutions of glycerol-methanol solutions of azure eosinate vary from about 1:35 to 1:52 in volume dilution.

CONCLUSIONS

The presence of any considerable amount, over 4 per cent, of methylene violet is tolerated in Romanovsky staining only in "on slide" staining with relatively high initial concentration of methanol in the final staining mixture. With concentrations below 20 per cent, such as are reached in Giemsa type methods, not much over 4 to 5 per cent methylene violet is tolerated. Larger amounts occasion precipitation of both the methylene violet and the thiazin eosinates. However, in "on slide" staining with methanol concentrations of from 30 to 40 per cent, the presence of from 15 to 30 per cent methylene violet distinctly improves azure eosinate staining. For all concentrations of methylene violet, from 25 to 33 per cent initial methanol concentration appears to be the best range for "on slide" staining. Higher concentrations give poorer staining with low methylene violet content in the dye; lower concentrations occasion precipitation with higher methylene violet levels. The latter limitation is the less important as the usual Wright stains appear to contain less than 7.5 per cent methylene violet, and this amount is tolerated even by a 20 per cent dilution of the methanol stock solution.

Alkali polychroming methods are difficult to control so as to produce constant proportions of the various azures, and they also produce a variable amount of methylene violet. Acid chromate polychroming is easily controlled, yields relatively constant products, and produces little or no methylene violet. It permits the use of the commercial double zinc chloride as well as of medicinal methylene blue.

Acid chromate polychroming with 200 mg. $K_2Cr_2O_7$ (or its Na equivalent) per gram of 86 per cent medicinal methylene blue (20 per cent less $K_2Cr_2O_7$ for the zinc salt) yields chiefly azure B with some methylene blue. This gives good staining of blood protozoa as well as of blood cells. Its eosinate is relatively more stable in methanol solution than eosinates containing higher proportions of azure A. The use of a 1:24 dilution of a 1 per cent solution of such an eosinate in equal volumes of glycerol and methanol can be highly recommended for blood staining. Further, this glycerol-methanol solution is much more stable than plain methanol solutions and can be used as a stock solution for dilution with methanol to 150 or 200 mg. per 100 c.c. for the usual "on slide" Wright staining technique.

Acknowledgment is due Medical Technician Daisy Plotka and Junior Chemist Dorothy C. Peterson for aid in staining and spectroscopic procedures.

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SEROFLOCCULATION WITH A NEW GROUP OF ANTIGENS IN SOME PATHOLOGIC CONDITIONS

"Pathognostic Seroflocculation"

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POSITIVE reactions with serodiagnostic tests for syphilis have been reported occasionally in diseases other than syphilis. Among the conditions which give such so-called false positive reactions, Stokes[1] mentions frambesia tropica, recurrent fever, trypanosomiasis, spotted fever, leprosy, scarlet fever, ulcus tropicum, endocarditis, septicemia, malaria, ulcus molle, lupus erythematosus, leukemia, pernicious anemia, mycosis fungoides, tuberculosis, anesthesia, malignant tumors, psoriasis, pemphigus, aphthae, Graves' disease, plumbism, diabetes, eclampsia, leishmaniasis, pellagra, beri-beri, and pregnancy.

With the refinement of the technique of the tests and with the improvements introduced in the preparation of the beef heart antigens, the number of conditions which produce so-called false positive reactions have decreased considerably. A list compiled by Eagle[2] in 1937 contained only six such diseases: trypanosomiasis, relapsing fever, infectious mononucleosis, malaria, leprosy, and yaws. Recently, however, with the widespread routine performance of serodiagnostic tests for syphilis, attention was again drawn to the occurrence of positive reactions in the absence of syphilitic infection. Eagle,[3] in 40,545 white students of both sexes in 25 universities, found 36 false positive reactions. Other workers (Moore, Eagle, Mohr[4]) reported false positive reactions in acute infections of the respiratory tract including pneumonia, and after smallpox vaccination (Lynch et al.[5]). The problem of the specificity of the syphilitic serodiagnostic tests was further complicated by the discovery that sera of normal animals give positive reactions (Porro,[6] Greene et al.[7]).

According to Kahn,[8] the true positive reactions can be distinguished from false or nonspecific reactions of human and animal sera by a special verification test in which different temperatures and salt concentrations for each type of reaction are used. True positive reactions are stronger at 37° C. and weaker at 1° C., while nonspecific reactions are weaker at 37° C. and stronger at 1° C. Specific serologic reactions tend to become stronger, and nonspecific weaker as the salt concentration of the reacting system is increased. Kahn[9] was able to obtain a very high percentage (95-98 per cent) of positive nonspecific reactions in Kahn negative sera by performing the test at low temperature, with unheated sera and with the use of a special sensitive antigen prepared from beef heart powder which was kept at 37° for several months. Based on these facts, Kahn recognizes the existence of a universal biologic type of reaction which can be dis-

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Received for publication, July 22, 1944.

covered under proper conditions, which is non-specific in nature and has no connection with syphilitic infection.

It is generally assumed that syphilitic sera contain a substance designated as reagin which produces flocculation in colloidal suspensions of beef heart extracts. Many investigators (Barnett et al.,[10] Sherwood et al.,[11] Lund[12]) believe that the so-called false positive reactions are due also to the presence of small amounts of reagin-like substances in nonsyphilitic sera. The opinion has, therefore, been expressed that the difference between true and false positive reactions is of a quantitative rather than a qualitative nature (Kolmer[13]).

From this short survey, it follows that up to the present the investigations of serodiagnostic tests were mainly concerned with syphilis. All the other pathologic conditions giving false positive reactions were regarded as side issues and received attention only insofar as they interfered with the differential diagnosis of syphilis. In any case, the low incidence of false positive reactions in each pathologic group permitted no definite conclusions as to their significance. Nevertheless, the fact remains that some pathologic non-syphilitic sera have a tendency to give positive flocculation tests.

It seemed worth while to detach the problem of serofloccculation from syphilis and explore the possibility of finding antigens which will bring out the tendency of serofloccculation in various other pathologic conditions in a constant and distinct way. It was clear that cholesterinized antigens prepared from beef heart would not serve this purpose since the standard antigens rarely gave positive reactions outside of syphilis, while the antigens prepared by Kahn from aged beef heart powder approached universal sensitivity by giving flocculation with nearly all sera, normal and pathologic alike.

In quest for a suitable antigen, we first tested alcoholic extracts prepared from various dry substances containing lipoid materials. They were milk powder, egg yolk powder, soya bean flour, jack bean meal, wheat flour, pulverized walnuts, almonds and dried yeasts. These extracts fortified by cholesterol gave positive flocculation reactions in some pathologic conditions but in a casual and non-uniform way, and therefore were discarded. In continuing our search for new sources, we finally selected the following substances for the preparation of antigens: I. *Animal Origin*—butter, chicken fat, human fat, lanolin (anhydrous), oil of lard, neat's-foot oil, sperm oil, suet; II. *Plant Origin*—oil of sweet almonds, castor oil, cocoanut oil, cottonseed oil, hydrogenated plant oil (Crisco), linseed oil, olive oil, peanut oil, poppy seed oil, sesame oil, soya bean oil, oil of walnut; III. *Mineral Oil* (Petrolatum liquidum).

TECHNIQUE

A. *Preparation of the Antigen.*—Stock solution: 1 ml. of oil or fat (when necessary after melting) was added to 100 ml. of absolute alcohol and the mixture was vigorously shaken. If some turbidity appeared, the mixture was placed in a water bath at 56° C. for 15 minutes and cooled at room temperature. When a sediment formed, it was removed by filtration. By addition of absolute alcohol to the stock solution, concentrations of less than 1 per cent could be prepared. The optimal concentration of the stock solution to be used in the test was determined by titration (see under *D*). We prepared alcoholic solutions from all the various substances listed above and tested each of them. Since, generally,

with few exceptions they reacted equally, we selected for the standard test one representative of each group: Lanolin, sesame oil, and paraffin oil. 0.75 ml. of the selected stock solution was mixed in a test tube with 0.75 ml. of 0.3 per cent cholesterol solution in absolute alcohol and 8.5 ml. of distilled water were added. For smaller series of tests, correspondingly lesser quantities of ingredients were used. The resulting suspension was milky, homogeneous, stable and without microscopically visible particles. No ripening was necessary. It was stored at room temperature and was serviceable for two to three days. A suspension prepared from the stock solution of oil without addition of cholesterol, just as the one prepared from cholesterol solution without stock solution of oil had no properties of an antigen.

B. *Preparation of Sera.*—Sera were inactivated at 56° C. for ½ hour. Unheated sera were not satisfactory for the test.

C. *Performance of the Test.*—Slides with cavities of paraffin rings were used. Into each cavity or ring 0.05 ml. of the antigen suspension was introduced and then the same amount of serum to be tested was added. The slide was rotated for 2 to 3 minutes. Positive sera showed clumps and flocculi which in most cases were visible with the naked eye. For verification, microscopic examination at low power with light cut down was used. In negative sera, no aggregation of particles took place.

D. *Titration of the Antigen.*—Five positive and five negative sera obtained by testing with undiluted stock antigens were selected for titration. The stock solution was diluted with absolute alcohol to form dilutions of 1:2, 1:4, 1:8, 1:16. From each dilution a suspension of the antigen was prepared by adding cholesterol solution and water. The positive and negative sera were retested with the suspension of each dilution. The highest dilution of the antigen which gave clear cut results with the positive and negative sera served as a titer and was used for the standard test. Usually the titer ranged between undiluted stock solution and the dilution 1:4.

RESULTS

The sera for investigation were obtained from patients in the wards and clinics of our hospital and also from many physicians to whom I am greatly indebted. In the group of normal sera were included specimens of premarital negative Wassermann sera kindly sent to me by several laboratories.

The testing of the sera with the new antigens gave the results listed in Table I.

TABLE I

	NUMBER OF SERA	POSITIVE		NEGATIVE	
			%		%
1. Normal sera (of presumably healthy persons)	665	14	(2.1)	651	(97.9)
2. Pneumonia	64	58	(90.6)	6	(9.4)
3. Other acute infections (meningitis, acute osteomyelitis, cellulitis, septicemia)	27	24	(88.9)	3	(11.1)
4. Acute coronary thrombosis	44	39	(88.6)	5	(11.4)
5. Pregnancy	177	15	(8.5)	162	(91.5)
6. Tuberculosis	271	190	(70.0)	81	(30.0)
7. Malignancy	263	221	(84.0)	42	(16.0)
8. Miscellaneous (non-infectious conditions—surgical, orthopedic, metabolic, etc.)	785	41	(5.2)	744	(94.8)
9. Syphilis (4 plus Wassermann)	124	45	(36.3)	79	(63.7)

Comparison With the Blood Sedimentation Rate.—The conditions, mentioned in Table I, which gave positive results with the new antigens are characterized usually by high blood sedimentation rates. In 82 cases both tests were performed—the seroflocculation and the blood sedimentation. The results are shown in Table II.

TABLE II

COMPARISON OF THE SEDIMENTATION RATE AND THE SEROFLOCCULATION

SEDIMENTATION RATE (WESTERGREN INDEX)	SPECIMENS	FLOCCULATION TEST	
		POSITIVE	NEGATIVE
Below 20	25	6	19
20-30	15	7	8
30-40	10	1	9
40-50	7	5	2
Above 50	25	18	7

DISCUSSION

The substances selected for the preparation of the new antigens comprise a group of fats and oils of animal origin, plant origin, and paraffin oil. These substances, in spite of their different chemical make-ups, have one feature in common. When dissolved in alcohol and mixed with water, they form milky homogeneous stable emulsions. From tests with these new antigens, the following conclusions can be made: (1) Sera of presumably healthy persons with few exceptions give negative results. (2) In a large majority of acute infections, especially pneumonia, and also in tuberculosis, cancer and coronary thrombosis, the test gives positive results. (3) Cases of pregnancy give a higher percentage of positive results than normals, but still far below the pathologic conditions just mentioned. (4) Among a heterogenous group of various non-infectious diseases, the percentage of positive results is only slightly increased. (5) Four plus Wassermann positive sera very often give negative results (63.7 per cent). (6) No complete agreement exists between the seroflocculation test and the blood sedimentation rate. The seroflocculation may be negative in cases with a high blood sedimentation rate and positive in cases with a low rate. In view of the tendency of the new antigens to produce flocculation reactions with sera in some pathologic conditions more or less constantly, we propose for this type of seroflocculation the name "pathognostic seroflocculation."

The test with the new "pathognostic" antigens presents some distinct features. First, as is known, very fine suspensions of cholesterinized beef heart antigens, especially those prepared with distilled water, react very weakly when added to syphilitic sera and produce small aggregates which become visible only after a lapse of several hours. In contradistinction to that, the finely dispersed watery suspensions of the new antigens when added to the corresponding pathologic sera give rise within 2-3 minutes to a very marked flocculation. Second, unlike the biologic type of Kahn test, especially with his newly described antigen of the universal sensitivity, the pathognostic flocculation is stronger at room temperature and 37° than at 0°, is rarely obtained with unheated sera, is negative in most of normal sera, and in a relatively large proportion of Wassermann positive syphilitic sera. All this suggests that the pathognostic seroflocculation might be a new phenomenon which is independent of syphilitic as well as of the biologic seroflocculation reactions. Since positive Wassermann and Kahn

sera containing reagin often fail to react with the new antigens, it may be assumed that the factor producing pathognostic seroflocculation is of a nature other than reagin. Furthermore, due to differences in the results of the blood sedimentation rate and the seroflocculation test, there is apparently no common factor responsible for both tests. In this first stage of our investigation, all our efforts were devoted to devising the test, selecting the antigens, perfecting the details of the technic and studying the general aspects of the phenomenon of "pathognostic seroflocculation." So far, no attempt was made to correlate the results of the test with the clinical findings, the duration of the disease, severity and extent of the process, complications or type of treatment. At present, it can be stated that a positive test suggests the presence of infection, inflammation or malignancy. It remains for further studies to investigate the various problems connected with the test and to find out its practical value for diagnostic or prognostic purposes.

SUMMARY

New antigens were prepared from paraffin oil and from fats and oils of plant and animal origin. The technique of testing sera with these antigens is described. The test gives negative results with sera of healthy persons and positive results in a majority of cases of acute infections, especially pneumonia, and in tuberculosis, cancer and coronary thrombosis. Four plus Wassermann and Kahn positive sera often fail to react with these antigens. The differences between this type of seroflocculation and the biologic and syphilitic type are discussed. The name "pathognostic seroflocculation" is proposed for the test.

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"REVERSE" TYPING AS AN AID IN THE DIAGNOSIS OF PNEUMONIA

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THE clinical similarity between atypical pneumococcic pneumonia and sporadic cases of primary atypical pneumonia makes a differentiation very difficult.[1] Pneumococcic pneumonia may often fail to show the invasive organism in the sputum. Several mechanisms could be responsible for this. If the pneumonic lesions are small and scattered, they may not communicate with a larger bronchus, similar to miliary tuberculosis of the lung; the communicating bronchus may be blocked by a mucous plug; the cough reflex may be suppressed in some patients; and finally, the sputum may contain inhibitory factors or bacteriolysins which may prevent satisfactory typing of the organisms. It is also well known that in children with pneumonia a satisfactory sputum specimen is frequently unobtainable. In these cases a method which would not depend on the bacteriologic sputum studies would be of diagnostic value.

About 70 per cent of patients with pneumonia develop specific antibodies between the seventh and tenth day of illness as determined by the passive protection of mice.[2] A simple method has therefore been adopted to utilize these antibodies diagnostically.

Method.—Fifty-six strains of pneumococci were prepared as follows: The virulent strains were passed through mice and grown in a 1 per cent bactopeptone broth containing from 5 to 10 per cent horse serum. After eighteen hours' growth, the cultures were centrifuged and the sedimented bacteria washed with saline. Saline suspensions of the recentrifuged organisms were then incubated in a water bath at 56° C. for one hour. Final dilutions containing 1 per cent formalin and about 0.2 mg. bacteria (dry weight) per cubic centimeter were made. The pneumococci of these suspensions showed distinct capsule swelling when mixed with a drop of specific antiserum containing from 5 to 10 units antibody per cubic centimeter. The strains were pooled in groups of six to simplify the diagnostic proeedure.

Using the patient's serum as antibody, the pooled strains were examined for quellung reaction as in the standard Neufeld method. If a patient's serum gave negative results at the onset of disease, and specific capsule-swelling antibodies in rising titers appeared later, the test was considered positive.

Agglutinin titers were determined on all specimens of serum which were typed by this method. In all instances the results of the agglutination reaction confirmed those obtained by the Neufeld reaction.

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Received for publication, May 3, 1944.

Results.—Eight of twenty-one patients* with pneumonia whose sputum could not be typed, in spite of repeated attempts, developed type-specific antibodies as demonstrated by the "reverse" typing method. The following types were encountered: I, II, III, VII, VIII, XXIII, XXVIII.

Summary and Conclusions.—By means of the "reverse" typing procedure, an etiological diagnosis may be made in certain patients with pneumococcic pneumonia in whom sputum studies are negative.

The authors wish to thank Miss J. Proctor for her technical assistance.

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*These patients received sulfonamide therapy as soon as a diagnosis of pneumonia was made. Sulfonamides do not interfere with the detection of capsule-swelling antibodies in the patient's serum.

A SIMPLE VENTILATION RECORDER FOR ANIMAL EXPERIMENTS

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THE metabolimeter is usually used for the accurate recording of respiration. In order to obtain the ventilation (cubic centimeters per minute), it is necessary to determine the depth of each inspiration in cubic centimeters and add these values. By a simple adaptation of the metabolimeter we have been able to record directly the minute ventilation as well as the rate and depth of respiration. Due to the simplicity of operation and interpretation of the records obtained with this apparatus we have also found it a valuable asset in the laboratory courses in physiology and pharmacology.

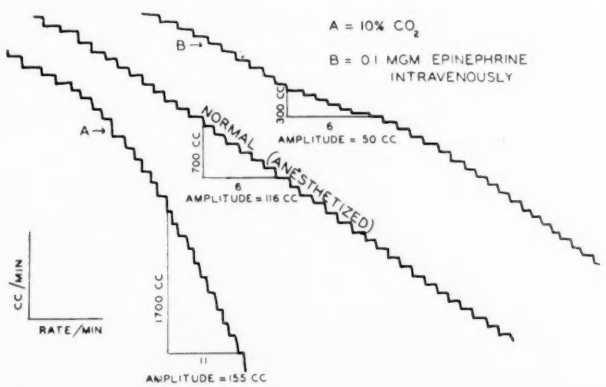

Fig. 1.—Ventilation response of the anesthetized dog to carbon dioxide and to intravenous epinephrine. Anesthesia—pentobarbital sodium.

Any standard metabolimeter may be adapted. The soda lime cannister is removed; the inflow valve is also removed and the opening is plugged with a rubber stopper. The outflow valve is left in place and is connected to a tracheal cannula or catheter by means of rubber tubing. The tracheal cannula is also connected to a water valve, which can easily be made from two "T" tubes which are connected with rubber tubing and with a clamp for opening and closing; the side arms of the "T" tubes project through a rubber stopper into a bottle containing a small amount of water. One side arm is cut off just below the stopper and the other one, which is connected to the tracheal cannula, is permitted to dip into the water. This valve furnishes just enough resistance so that inspiration is from the metabolimeter and expiration is into the room air. The clamp should be opened when the metabolimeter is being filled. The apparatus is quickly filled by removing the rubber stopper on the metabolimeter and raising the metabolimeter bell.

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Received for publication, July 10, 1944.

This arrangement gives a "stair-step" record of ventilation. Inspiration is represented by the vertical lines and expiration by the horizontal lines (see Fig. 1). We have found it convenient and cheap to substitute a good grade of wrapping paper for the usual metabolimeter paper. Quantitative analysis of the record is accomplished by means of a ruler whose width represents the distance moved by the metabolimeter drum in one minute; the length being marked off in cubic centimeters representing the volume of the metabolimeter. This information is easily obtained from the metabolimeter paper used for the apparatus. We have found x-ray film a convenient material to use for the ruler.

In Fig. 1 a typical record obtained by students is reproduced. The changes in ventilation are obvious because of the changes in the slopes of the lines. Analysis of the record reveals that the response to epinephrine in this dog consisted of a change in depth of repiration with a marked reduction in the minute volume of respiration. The usual response to carbon dioxide is also shown with a marked increase in rate, depth, and minute volume.

MEDICAL ILLUSTRATION

AN INEXPENSIVE METHOD FOR PUBLISHING PHOTOGRAPHS OF FLUORESCENCE PHENOMENA IN COLOR

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IN A PREVIOUS paper a method for recording fluorescence phenomena on Kodachrome film was described.[2] Although this method reproduces the various fluorescent colors, the cost of publishing many of these pictures by the three or four-color process is prohibitive. A method was, therefore, devised for photographing fluorescence on black and white film in such a manner that reproductions of fluorescence were obtained which were suitable for printing in color by the Ben Day process. The resulting illustrations are useful to depict one or possibly two fluorescent colors. If only one color is pertinent, it may be emphasized and accurately recorded by this relatively inexpensive method.

One of the requirements for superimposing a color on a black and white illustration by the Ben Day process is that the area to be colored must be almost white. This effect is produced by great overexposure of the negative to the fluorescent light of the color which it is desired to record or emphasize. For example, if red fluorescence is to be recorded, the negative is overexposed to the red fluorescence by the use of a red filter so that red fluorescent tissues are recorded as nearly opaque areas on the negative. On the positive print these show up as almost white fields upon which the corresponding color can be superimposed by the Ben Day method. The precise color of the fluorescence can be described to the engraver or printer in terms of conventional color charts or notations,[3] or the color can be matched by an artist. Printers' inks can be selected to match this color and thus accurately represent the fluorescence.

The illustration of the red fluorescence of the harderian gland of the rat (Plate I) was chosen to permit comparison of this new inexpensive method with the Kodachrome four-color method described previously in the JOURNAL.[2] A picture of the fluorescence of the rat and the harderian gland (Fig. 2, Plate I) in black and white is also shown. This is even less expensive than the process by which the Ben Day color process is applied but not nearly as satisfactory. The added color for purposes of depicting or emphasizing the fluorescence in a certain color costs but little more (Fig. 4, Plate I). The general principles and precise steps necessary to produce these illustrations are outlined.

The ultraviolet light sources and general principles of the use of filters for photographing fluorescence have been described.[1, 2] For Fig. 1 the rat with the harderian gland exposed was arranged on black paper. A 100-watt near-ultraviolet lamp and two 100-watt tungsten bulbs were placed on each side. A 500-watt tungsten lamp on a movable reflector was used to illuminate from

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This work was aided by grants from the Anna Fuller Fund for Cancer Research, the International Cancer Research Foundation, and the Bressler Alumni Research Fund.
Received for publication, July 6, 1944.

different angles during the exposure to eliminate shadows. All the lights were turned on and the Super XX film was exposed for 40 seconds at f:45 without a filter.

In Fig. 2 the rat was photographed in a similar manner, except that no visible light was used and a Corning filter No. 3060 Noviol was interposed between the film and the object. A Wratten 2A filter would have been equally satisfactory. The rat was thus illuminated by only two near-ultraviolet lamps, one on each side. The Super XX film was exposed for 4 minutes at f:16. This gave, in reality, a photograph of the fluorescence on black and white film which has been reproduced as such. It may be seen that this type of picture is not suitable for the illustration of the red fluorescence of the harderian gland because the gland appears too dark to superimpose the red on it.

To obtain the photograph of the rat in Fig. 3 a red filter (WrattenF29) was arranged in a Universal swivel clamp so that it could be rotated into position in front of the lens after the film had been partially exposed with visible light. The Super XX film was then exposed by using the tungsten lights as well as the two near-ultraviolet lights for 12 seconds at f:45. This was only one-third of the exposure time which had been used for Fig. 1. The visible lights were then turned off and the red filter moved over in front of the camera lens. The exposure was then continued for 45 minutes at f:5.6. The red filter permitted the light from the red fluorescent gland to pass through and thus overexposed this area on the negative. It also transmitted the less intense red from other areas and completed the proper exposure of these areas. During the entire exposure the film was shielded from near-ultraviolet light by a Corning filter No. 3060 Noviol placed behind the lens. The red Wratten F29 filter also prevented near-ultraviolet light from reaching the film during the last 45 minutes of the exposure. Duplicate prints of photographs were made to submit to the engraver. One of these was colored to indicate the areas where Ben Day color was to be applied. The other black and white copy was necessary to make the engraving upon which the Ben Day color was superimposed (Fig. 4).

Fluorescence of other colors may be recorded and illustrated in a similar manner by choosing appropriate filters and film. In general, the film should be relatively sensitive to the color of the fluorescence to be recorded. Panatomic X or Super XX are satisfactory for red. The color sensitivity spectrograms have been found helpful in selecting film for recording various colors.

PLATE I

Four photographs of a normal rat with the left harderian gland exposed by the removal of the eyeball. Eastman 5 by 7 inch view camera, 6½ inch Goerz Dagor lens.

Fig. 1.—Illumination: four 100-watt stationary incandescent tungsten filament bulbs, one moving 500-watt incandescent tungsten filament at approximately 3 feet, and two G.E. BH 4 100-watt quartz mercury arc near-ultraviolet lamps. Super XX film; no filter. Exposure time, 40 seconds at f:45.

Fig. 2.—Same as Fig. 1, except visible lights turned off. Illumination: two G.E. BH 4 100-watt mercury arc near-ultraviolet lamps. Secondary or lens filter: Corning No. 3060 Noviol. Exposure time, 4 minutes at f:16.

Fig. 3.—First exposure illumination: visible light same as Fig. 1 for 12 seconds at f:45. Secondary filter: Corning No. 3060 Noviol. Second exposure illumination: two G.E. BH 4 near-ultraviolet lamps only. Additional secondary lens filter: Wratten F29. Exposure time, 45 minutes at f:5.6.

Fig. 4.—Duplicate of photograph in Fig. 3 with red fluorescence of harderian gland indicated by Ben Day process. Compare with Fig. 2A, Plate I, Figge and Clarke, this Journal, 27: 1609, 1942.

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Fig. 1.

Fig. 2.

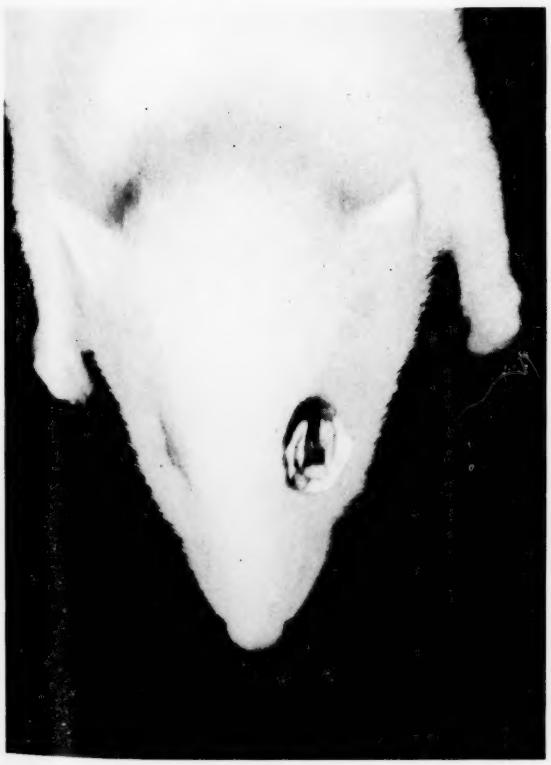

Fig. 3.

Fig. 4.

PLATE I.

(See opposite page for legend.)

The Wratten tricolor filter sets are ideal for transmitting fluorescent light of any given color to overexpose the film. The following will be found satisfactory for any definite blue, green, or red fluorescence:

Wratten No. 49 filter C4 for blue
Wratten No. 61 filter N for green
Wratten No. 29 filter F for red

Only in rare instances will it be necessary to use filters that transmit wider bands of light. A hand spectroscope is excellent for determining the fluorescent spectrum so that a filter which specifically transmits these wave lengths may be selected. The only advantage to be gained by such precision would be possibly to shorten the exposure time in some instances. The intensity of the fluorescent light is usually so low that the exposure time cannot be determined by means of an ordinary exposure meter. Even with an extremely sensitive meter this is somewhat impractical, because the exposure time is so long, and excessive overexposure to fluorescent light from most objects is almost impossible.

If it is necessary to depict two fluorescent colors on one object, the film may be overexposed to each color separately and the areas for each color indicated. The Ben Day printer can place the colors to correspond to those observed on the object. Another alternative would be to make two photographs to be printed together, each depicting the fluorescence of one primary color.

SUMMARY

A method for photographing fluorescence on black and white film is described. The resulting photograph may be published in color by the relatively inexpensive Ben Day process.

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BOOK REVIEWS AND NOTICES

Medical Diagnosis. Applied Physical Diagnosis. By *Roscoe L. Pullen*, A.B., M.D., Instructor in Medicine, Tulane University of Louisiana School of Medicine; Assistant Clinical Director, Charity Hospital of Louisiana at New Orleans; Formerly Fellow in Clinical Endocrinology, Duke University School of Medicine and Duke Hospital, Durham, N. C. With a foreword by *John H. Musser*, B.S., M.D., F.A.C.P., Professor of Medicine, Tulane University of Louisiana School of Medicine; Senior Visiting Physician, Charity Hospital of Louisiana at New Orleans. W. B. Saunders Co., Philadelphia and London, 1944. Cloth with 1,106 pages. 584 illustrations, and 12 color plates.

This new textbook of physical diagnosis differs in at least two ways from other similar books now available. First, it was written by twenty-six clinicians in addition to the editor; there are many evidences, therefore, of the specialized information one would expect when authorship is the work of a group of physicians rather than of one. Second, it devotes chapters to techniques of examinations which are usually treated very briefly in textbooks of physical examination. There are, for instance, detailed discussions of proper methods for examining the skin, the eyes, the ears, nose, and throat, the oral cavity, the breasts, the urogenital tract, and the anorectal region. Methods for making an endocrine survey are described. Chapters are devoted to psychiatric diagnosis, to special problems of pediatric diagnosis, to occupational injuries, and to military problems. Endoscopic techniques, like proctoscopy, are described in considerable detail. Description of these methods gives the book special value to physicians as a reference source. Hospital house staffs should find them most useful.

In most of the sections, material is presented in a very readable style. Illustrations are clear, well chosen, and really instructive. The chapter on the examination of the extremities is particularly well done. One wonders, however, at the wisdom of providing medical students with a textbook on physical diagnosis which devotes only sixty-four pages to examination of the chest, but forty-five to examination of the breasts and ninety-eight to gynecologic and obstetric diagnosis. There are certain other inconsistencies. Thirteen positions are given in which the breasts may be studied, but no mention is made of the fact that spleens may often be felt more readily when a patient lies on his right side with the left thigh flexed on the abdomen. The inclusion of histologic findings under conditions where the changes do not contribute to an understanding of the attendant physical signs is disconcerting. The same objection applies to the listing of certain hematologic data in a discussion of enlarged lymph nodes.

These criticisms, however, are not meant to imply that the book is not an unusually serviceable one. It will undoubtedly be adopted as the standard text in many medical schools. For the general practitioner, the internist, and the surgeon, the special chapters already mentioned will be particularly valuable guides.

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